Food Research

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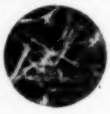

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STUDIES ON ENZYMATIC TENDERIZATION OF MEAT. II. PANEL AND HISTOLOGICAL ANALYSES OF MEAT TREATED WITH LIQUID TENDERIZERS CONTAINING PAPAIN[a]

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(Manuscript received March 22, 1958)

Proteolytic enzymes from plant sources have been used for several years to tenderize meat. An early attempt to understand something of their mode of action was reported in 1942 by Gottschall and Kies *(1)*. They found that the most rapid digestion by papain occurred between 158 to 185° F. with very little taking place below 86° F. The papain did not penetrate further than 2.5 mm. below the surface of raw beef at room temperatures. The activity of this surface coating of enzyme was quickly lost upon exposure to cooking temperatures above 212° F. Thus, it was necessary for the enzyme to penetrate the meat before the cooking process began. Powdered enzyme preparations augmented by physical treatment to facilitate penetration of the meat *(2)* were shown to increase the tenderness of steaks 0.5 to 2.0 inches in thickness. Holding the treated meat at room temperature for as long as five hours before cooking had no effect upon the extent of tenderization *(4)*. Tissue changes near the surface included destruction of the sarcolemma and muscle fiber nuclei and disappearance of the collagenous fibers. Preliminary studies on the use of a liquid tenderizer in this laboratory have been reported *(8)*.

The basic problem in using enzymes to tenderize meat appears to be that of obtaining uniform enzyme distribution. Commercial meat tenderizers of the liquid type containing papain dissolved in concentrated salt solution and including generally, hydrolyzed vegetable protein, present a possible means of assisting enzyme penetration. In these experiments the influence of tenderizing agents on the structure of the muscle tissue as determined histologically has been correlated with the effects noted by panel evaluations.

MATERIALS AND METHODS

General design. Entire ribeye muscles of utility grade beef cow carcasses were chilled to 28° F. and sliced into ¾ inch steaks. Steaks were immersed in tenderizer solution at 40° F. for ½ minute, drained on a stainless steel rack, wrapped and tightly sealed in MSAT-80 cellophane. The steaks were weighed before and after treatment with tenderizer. Control steaks were wrapped without treatment. Steaks were found to pick up about 1% their weight when dipped in water under conditions similar to the tenderizer treatment. This moisture appeared to be lost during further handling (freezing and thawing), therefore the control steaks for this study were untreated. Most of the steaks were placed in a −20° F. room immediately after wrapping, others

[a] Journal paper No. 160, American Meat Institute Foundation, Supported in part by a grant from Huron Milling Company, Harbor Beach, Michigan and Paul-Lewis Laboratories, Inc., Milwaukee, Wisconsin.

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were held at 40° F. for 2 hours before freezing. Steaks were stored at −10° F. and laboratory tests were completed within two weeks of treatment. Three liquid tenderizers containing papain were tested. Two of these, A and B, contained hydrolyzed vegetable proteins and approximately 14% salt expressed as sodium chloride; the other one, C, contained more salt and no hydrolyzed vegetable proteins. Generally, the steaks were held at 40° F. for 16 hours before cooking to allow for complete thawing. A few of the steaks were cooked directly from the frozen state while others were thawed at 70° F. for 24 hours and cooked without freezing. Direct comparisons were made between treatments through the use of triangle tests. Three adjoining steaks were used for each test, the center steak being treated in one manner and the outer two in an alternate one. Each treatment was carried out on steaks from 3 animals.

Steaks were cooked in an electric broiler regulated so that the temperature, 4 inches distant from the element, was maintained at 450° F. The steaks were turned over when the internal temperature reached 120° F. and cooking continued until this temperature reached 150° F. Steak temperatures were measured using copper-constantan thermocouples. A record was kept of the weight of each steak before and after cooking and of the drip loss during cooking. From these figures the per cent yield of cooked steak and loss by evaporation during cooking were calculated. Samples of cooked steaks were used for panel and histological testing.

Panel Methods. The taste panel was comprised of 8 persons experienced in scoring beef for tenderness. All of the taste panel members examined portions from each of the steaks used in the treatment comparisons. Samples were arranged so that each taste panel member received samples from similar locations within adjacent steaks. The panel member was asked to select the sample which had been treated differently and to score all of the samples for tenderness. A scoring range of 1-10 was used, higher scores being given to more tender steaks. The choices made in triangle tests were tested for significance based on Chi-square tests. The scores for tenderness were analyzed for variance and the results expressed as "F" values *(3)*.

Histological. Both raw and cooked tissue were celloidin processed. Small tissue blocks, approximately 6 mm. in thickness, were fixed in 10% neutral formalin overnight, cut in celloidin at 10 microns, and triple stained with hematoxylin, Van Gieson's and Weigert's.

Early in the work, some samples from frozen-dried steaks were rehydrated in a commercial tenderizer solution for 30 minutes and subsequently similarly celloidin processed. This was done to find out what over-treatment with the liquid tenderizer might do structurally to the muscle tissue. None of these deliberately over-treated tissues were cooked.

The blendoring technique developed in our laboratory was used to advantage for studying the detailed structure of muscle fibers as well as determining their extensibility *(6)*. Isolated muscle fibers, together with adjoining connective tissues from samples of cooked, tenderizer treated steaks, were stained *in toto* with hematoxylin and Van Gieson's. Attention was directed to the sarcolemma and muscle fiber envelopes of those fibers obtained from control and treated steaks. These observations were made in the light of structural details seen in celloidin sections of the same samples.

Attempts also were made to trace the elastic tissue in treated samples by making sections with the freezing microtome and staining them with Weigert's stain. The thickness of these sections (40 microns) allowed a better perception of the continuity of the individual elastic fibers, but did not allow satisfactory photomicrography of the material.

In some experiments cooked tenderizer A treated and untreated steaks were subjected to dissection under the binocular microscope for a gross evaluation of the degree of connective tissue degradation. The purpose was to separate the muscle fiber bundles with forceps, thereby noting the relative amount of effort needed to break loose the perimysial strands of connective tissue (mostly collagenous) that bind the fiber bundles together.

RESULTS AND DISCUSSIONS

The steaks increased an average of 3% in weight when treated with the tenderizer preparations. The apparent amount of preparation adsorbed varied from 1.3 to 6.9%, depending somewhat upon the weight of the steak, but to

a greater extent upon the closeness with which the muscle bundles were bound together. Steak surfaces on removal from the freezer had the dark color typical of frozen beef. Unlike conventional frozen beef the meat treated with tenderizer did not regain, on exposure to air, the bright red color of fresh meat. Drip losses due to thawing were similar for all steaks varying less than 1 g. from the average of 1 g. per steak.

Results of the tests comparing preparations A, B, and C (Table 1) indicated that there was no appreciable difference in the tenderness of steaks treated with either tenderizer A or B. Steaks treated with tenderizer C which did not contain hydrolyzed vegetable protein and had a higher salt content, were much less tender than those treated with tenderizer A. Tenderizer A was used throughout the remainder of the study.

TABLE 1

A comparison of the tenderizer effect of three liquid commercial tenderizers

Tenderizer used	No. of samples judged	Triangle test selections		Tenderness scores	
		Correct	Incorrect	Mean	"F" value
A	24	3	21	7.3	0.17
B	48			7.5	
A	24	17[1]	7	7.0	90.28[1]
C	48			5.7	

[1] Denotes significance at 0.1% level

Panel Evaluation. The judges' selections in the triangle tests comparing untreated and tenderizer treated steaks were influenced by flavor as well as tenderness differences. This did not affect the use of triangle tests in those comparisons where all the steaks were treated (Tables 1 and 2). The treated steaks had a slightly bitter taste which was easily recognized by panel participants after the initial tasting session. The tenderness difference between treated and control steaks was so great (Table 3) that the influence of flavor differences upon the panel scores was slight.

Pre-cooking temperature affected the tenderness of treated steaks in some cases (Table 2). Although steaks maintained 24 hours at a refrigerator temperature of 40° F. were just as tender as those frozen for 6 hours at −10° F. and then held 18 hours at 40° F., steaks cooked without thawing were judged slightly less tender than comparable ones thawed at 40° F. for 18 hours. Unless enzyme penetration had occurred prior to cooking, it would be destroyed by the 450° F. broiler temperature and would explain the lesser effect on tenderness. Otherwise the more gradual temperature rise in the interior of the steak (Figure 1) would have resulted in a greater rather than lesser effect. The steaks thawed at room temperature (70° F.) for 2 hours were much more tender than those thawed for 18 hours in the refrigerator (40° F.) and steaks thawed at room temperature were mushy on the surface. Thus, the extent of tenderization produced by the preparation was not influenced by freezing, but was influenced markedly by temperature of thawing frozen treated steaks.

The tenderizer acted as effectively upon the steaks whether they were from muscles aged 48, 72, 96, or 120 hours before treatment with tenderizer (Table 3). Changes in the muscle during this aging period were without

TABLE 2

The effect of precooking storage temperature upon the tenderness of steaks treated with tenderizer A

Precooking storage		No. of samples judged	Triangle test selections		Tenderness scores	
Temperature	Time		Correct	Incorrect	Mean	"F" value
−10° F. 40° F.	2-6 days 18 hours	42	7	14	7.3	1.66
45° F. −10° F. 40° F.	2 hours 2-6 days 18 hours	21			6.9	
40° F.	24 hours	24	8	13	8.7	0.15
−10° F. 40° F.	6 hours 18 hours	48			8.8	
−10° F. 40° F.	6 hours 18 hours	21	8	12	8.2	5.53[1]
−10° F.	24 hours	42			7.4	
−10° F. 40° F.	6 hours 18 hours	21	12[1]	9	6.8	20.35[2]
−10° F. 70° F.	22 hours 2 hours	42			7.9	

[1] Denotes significance at the 5% level
[2] Denotes significance at the 0.1% level

apparent effect upon the tenderizing activity of the preparation. The slight increase in tenderness of the untreated samples may reflect normal aging processes. The fact that the increase in tenderness of the treated steaks was not as great as in the untreated ones supports this view.

The increase in tenderness due to treatment was observed whether the steaks were cooked by broiling or by pan frying. The cooking time was the same for both methods (Figure 1). Pan-fried steaks took 6 minutes to reach an internal temperature of 150° F; broiled steaks took 5 minutes when the steaks had been thawed at 40° F. Cooking losses (Table 6) were similar for both cooking methods, but pan frying resulted in a slightly

TABLE 3

The effect of the post-slaughter age of the meat upon the tenderizing effectiveness of tenderizer A

Post-slaughter age (hours)	Tenderizer treatment	Number of judgements	Tenderness scores	
			Mean	"F" value
48	Tenderizer A	24	8.0	94.57[1]
	None	48	4.9	
72	Tenderizer A	24	8.2	50.63[1]
	None	48	5.4	
96	Tenderizer A	24	8.3	75.20[1]
	None	48	5.8	
120	Tenderizer A	24	8.4	68.82[1]
	None	48	5.7	

[1] Denotes significance at 0.1% level

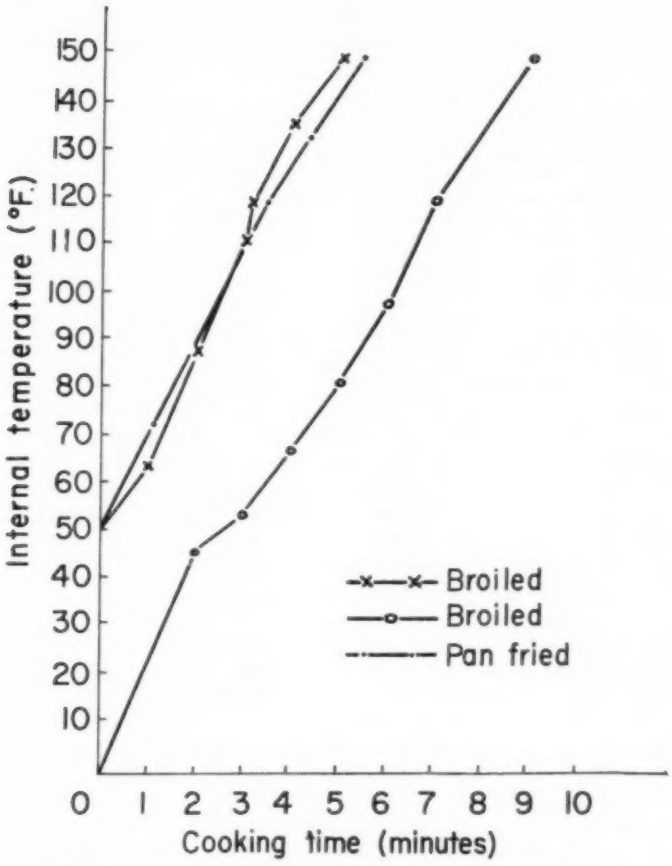


Figure 1. Internal temperature changes in steaks during cooking.

greater yield of cooked meat. Steaks cooked from the frozen state were exposed to broiler temperatures for 3 minutes longer during cooking than those cooked from refrigerator and room temperatures (Figure 1). They also had greater evaporation and total cooking losses (Table 5). The use of tenderizer did not affect the weight lost during cooking (Table 4). The slightly greater cooked weight of the treated steaks was due in part to the increased weight of the raw steaks treated with the tenderizer preparation.

Histological. Structural changes in raw beef. Treatment of raw steaks with tenderizer A by the usual dipping procedure did not result in any appreciable structural alterations, not even on the surface of the steaks. Increasing the intensity of treatment by using frozen-dried steaks, decreasing the sample size to blocks 5 mm. in thickness, and increasing the treatment time to 30 minutes by direct immersion in tenderizer A yielded different results. In this case, granulation invariably occurred in the interfibral spaces

TABLE 4

The effect of tenderizer treatment upon weight changes by steaks during cooking

Factor measured	Tenderizer treatment	
	Tenderizer A	None
Number of steaks	18	36
Raw weight (ave. g.)	171.4	171.7
Tenderizer (ave. g.)	6.3	0.0
Cooking loss—drip (ave. g.)	11.2	7.6
evaporation (ave. g.)	19.4	20.8
Cooked weight (ave. g.)	147.0	143.4
yield[1] (ave. %)	85.8	83.6

[1] Based on raw weight of steak

as typically shown in Figure 4. This was the only manifestation of the treatment; in all other respects, the tissue appeared indistinguishable from normal. The granulated material was ascertained to have been derived from endomysial collagen and the muscle fiber envelopes, both of which made early contact with the liquid tenderizer.

Structural changes in cooked beef. When treated steaks were broiled by the standard procedure, the structure was changed to that which is characteristic of the blocks of frozen-dried steak after 30 minutes immersion in the tenderizer (Figure 4). Many celloidin sections of these broiled fresh steaks were observed microscopically. It was difficult to distinguish them from celloidin sections of small blocks of frozen-dried steaks which had been immersed in tenderizer for 30 minutes. Both were characterized by an extensive granulation of the endomysial collagen. Higher magnification revealed that the sarcolemma and muscle fiber envelopes had been disintegrated (Figures 5 and 6) indicating that these structures may have been a precursor of the granular material.

This type of granulation is not the same as that which usually results from hydrolysis of collagen upon cooking. Figure 3 shows a photomicrograph made from a section of a cooked steak. The three arrows point to intact collagenous fibers which had not hydrolyzed. Most of the areas shown as the perimysial spaces are filled with an amorphous material (grayish in the photomicrograph; staining yellow with Van Gieson's in the actual preparation) which represents hydrolyzed collagen. This material did not have the same appearance as the granular material shown in Figures 4, 5, or 6 which resulted from treatment with tenderizer, either with or without the

TABLE 5

The effect of thawing procedure upon the weight changes during cooking of tenderizer treated steaks

Factor measured	Thawing conditions		
	Not thawed	16 hours at 40° F.	2 hours at 74° F.
Number of steaks	6	12	6
Pre-freezing weight (ave. g.)	123.8	129.2	133.4
Cooking loss—drip (ave. g.)	3.6	4.3	6.0
evaporation (ave. g.)	17.7	12.9	14.0
Total loss (ave. g.)	21.3	17.2	20.0
Cooked weight (ave. g.)	102.5	112.1	113.4
yield (ave. %)	82.8	86.7	85.0

TABLE 6

The effect of method of cooking upon weight changes in untreated and tenderizer treated steaks

Cooking method	Treatment	
	None	Tenderizer A
Broiling		
no. of steaks	6	3
raw weight (g.)	137.5	136.4
tenderizer (g.)	0.0	2.6
cooking loss—drip (g.)	5.3	5.0
evaporation (g.)	15.2	13.3
cooked weight (g.)	117.0	121.0
yield (%)	85.1	88.7
Pan frying		
no. of steaks	3	6
raw weight (g.)	164.0	164.0
tenderizer (g.)	0.0	2.7
cooked weight (g.)	146.3	146.7
yield (%)	89.2	89.4

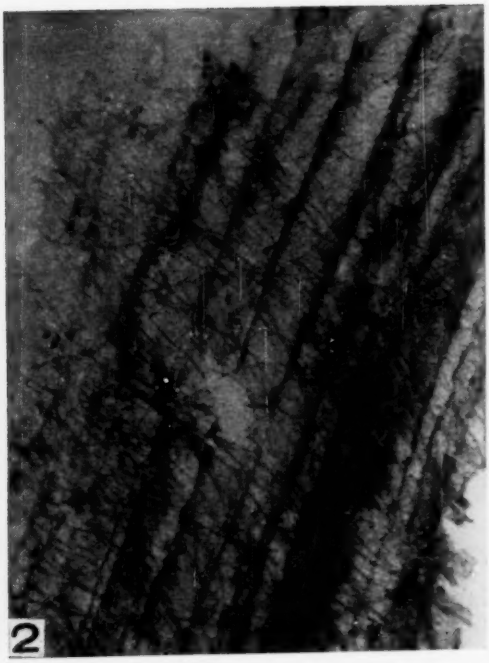
addition of heat. This implied that the end products resulting from heat and from the action of tenderizer were structurally different. The fact that tissue treated with tenderizer A yielded similar appearing granules whether or not followed by cooking, further indicated that the phenomenon of granulation was truly the result of the action of tenderizer and was not duplicated by heat.

The granulation was largely confined to the endomysial spaces and relatively small perimysial spaces. This was confirmed by dissection performed on the cooked treated steaks. Invariably, the larger perimysial strands of connective tissues (seen as definite white threads) which link adjacent sizable bundles of muscle fibers, were usually structurally intact, and not discernibly different in the control and experimental steaks. There was no difference in the amount of force required to pull apart these bundles in the two kinds of steaks.

No apparent effect on the elastic tissue could be observed from either celloidin sections or frozen sections except for a decrease in the staining capacity of the elastic fibers in cooked tenderizer treated samples. Since it is known that cooking does not visibly affect elastic fibers, this decrease may reflect either a "preconditioning" effect exerted by the tenderizer on these fibers, which would require the additional heat to bring about the decrease in staining capacity, or simply the continuing activity of the tenderizer during the early part of the cooking process.

Structure and extensibility of isolated muscle fibers. There was never an instance in which tenderizer A caused a visible change in the nuclei of either muscle fibers or free connective tissue cells or in the muscle fiber (Figures 4, 5, and 6).

Comparison of isolated muscle fibers from cooked control (Figure 7) and cooked treated samples (Figures 8, 9, and 10) have substantiated the fact that besides the endomysial collagen, which makes up the bulk of the granulated material, the sarcolemma and muscle fiber envelopes also contribute their share. Figure 7 shows several empty muscle fiber envelopes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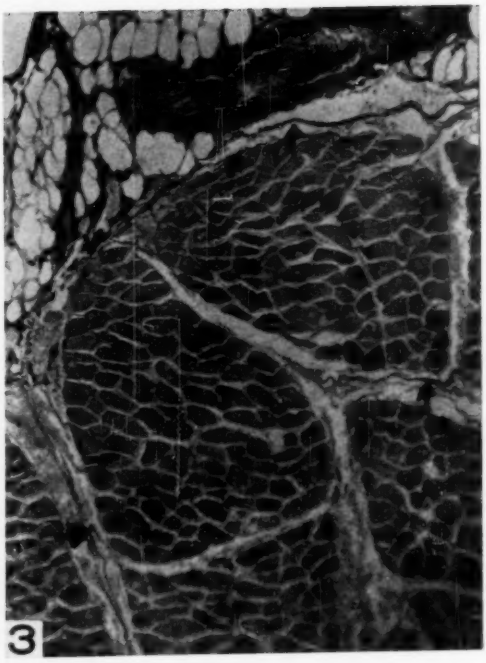
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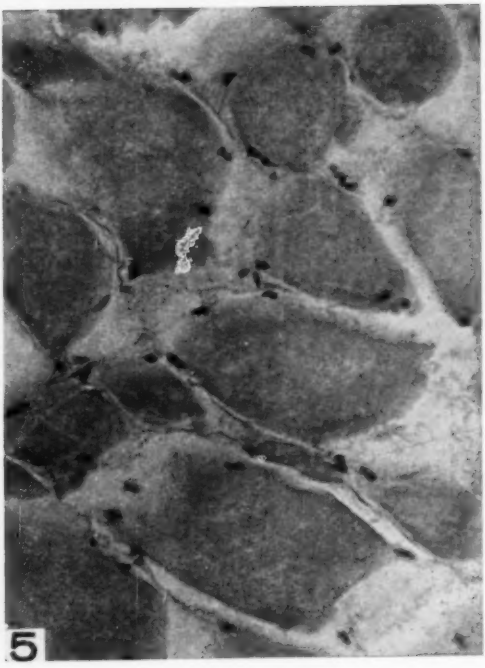
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and some adjoining connective tissues studded with nuclei and free cells. They were typical of blendored cooked beef, such as shown in Figure 2. Comparable material from a cooked treated sample is represented in Figure 9. Note the pattern of disorganization of the muscle fiber envelopes and endomysial connective tissue. In a section, this material would not look different from the granules shown in either Figure 4 or Figure 6. Figure 10 portrays the final stage in this process of granulation, and in a section, it would look like that shown in Figures 4, 5, or 6. That the sarcolemma also participates in the granulation is illustrated in Figure 8, in which spots of "eroded" sarcolemma are clearly indicated.

Muscle fiber extensibility has been shown to bear an inverse relationship to tenderness for some samples of meat *(6)*. Since an increase in tenderness was shown by the panel scores for the tenderizer treated steaks, much interest was attached to whether a difference in the extensibility of the muscle fibers might exist between the treated and untreated steaks. Many fibers such as those shown in Figures 8, 9, and 10 and those from their respective controls were measured. There were no differences in their average (20 determinations per sample) extensibility readings. Thus, tenderness in the samples studied here was not related to muscle fiber extensibility. Since the breakdown of the endomysial collagen and the disintegration of the sarcolemma and muscle fiber envelopes is related to the tenderness increase, it would appear that muscle fiber extensibility is a property of the actomyosin complex and is unrelated to the sarcolemma and muscle fiber envelopes.

It was previously shown that papain as an ingredient in a commercial meat tenderizer did affect the muscle fibers *(7, 8)*. However, in all the results presented here there were no indications of an affect on the muscle fibers, judging either from their structure or muscle fiber extensibility value. This suggests that either the papain concentration used was below the threshold level or that its action was modified by the other ingredients present (i.e., the hydrolyzed vegetable protein and salts). The combined action of the hydrolyzed vegetable protein and the salts dissolved in it is apparently responsible for the structural changes induced by the liquid tenderizer.

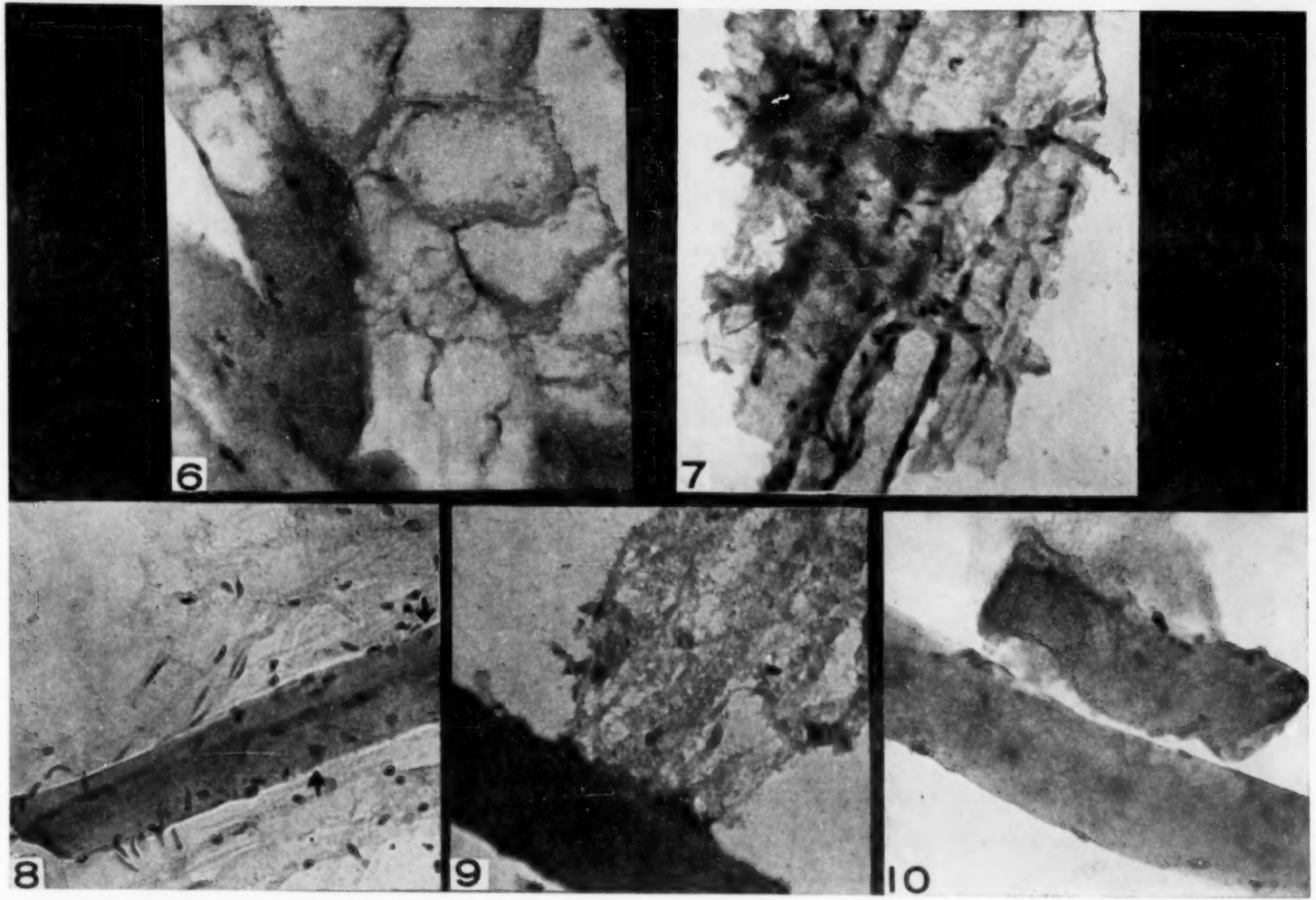
Effect of procedure variations. During the course of this study a number of variations in procedure were tested, e.g., chilling instead of freezing the steaks following tenderizer treatment and pan frying instead of broiling

Figure 2. A row of whole empty muscle fiber envelopes, isolated by blendoring, from cooked beef longissimus dorsi previously fixed in Zenker-formol. The material, stained with hematoxylin and Pollak's trichrome, shows the nuclei of connective tissue cells and a considerable amount of endomysial collagenous fibers intimately associated with the envelopes. × 150.

Figure 3. Ten-micron celloidin section of cooked longissimus dorsi showing two kinds of collagen, the hydrolyzed (gray) and intact (arrows) fibers filling the perimysial spaces. × 75.

Figure 4. Longitudinal section of frozen-dried sample of longissimus dorsi steak after direct immersion in tenderizer A for 30 minutes. Note extensive granulation throughout the interfibral spaces due to (a) degradation of endomysial collagen and (b) substantial disintegration of the sarcolemma and muscle fiber envelopes. × 75.

Figure 5. Celloidin section of cooked tenderizer A treated longissimus dorsi steak to show absence of sarcolemma and muscle fiber envelopes in most places and the characteristic endomysial granulation. × 320.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the treated steaks. Histological samples of steaks treated in these contrasting ways revealed no significant differences in the structure. Similarly, treated steaks thawed at various temperatures and for various lengths of time prior to cooking were structurally indistinguishable from one another. Steaks aged for varying periods (48-120 hours) before they were treated were also histologically similar. All of them were characterized by just one outstanding feature, namely, massive endomysial granulation, as described above.

In another experiment, tenderizer A was compared to two other commercial liquid tenderizers, B. and C. Tenderizer C caused a greater increase in the diameter of muscle fibers than the other two. Upon careful analysis of the muscle fiber diameter in isolated fibers immersed in varying concentrations of sodium chloride, it became apparent that the effects noted for tenderizer C were due to its higher salt concentration.

SUMMARY

Ribeye steaks were made more tender by treatment with liquid preparations containing papain, sodium chloride, and hydrolyzed vegetable proteins. There was no change in muscle fiber extensibility. The sarcolemma and muscle fiber envelopes were disintegrated and extensive granulation of the endomysial collagen occurred. The histologic changes were apparent in the cooked, but not in the raw steaks. The increase in tenderness and granulation brought about by the tenderizer solution was unaffected by aging the meat more than 48 hours before treatment or by holding at temperatures below 40° F. prior to cooking. The increase was more pronounced when frozen steaks were allowed to thaw before cooking. Thawing at 70° F. resulted in mushiness of the steak surface and a greater increase in over-all tenderness.

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Figure 6. Cooked treated muscle tissue showing very extensive granulation in a small perimysium with very little intact collagen left. × 320.

Figure 7. Several intact muscle fiber envelopes isolated from cooked, untreated longissimus dorsi, comparable to those shown in Figure 2. × 320. Both Figures 2 and 7 bring out the absence of heat effect on the material.

Figure 8. A whole muscle fiber surrounded with connective tissue, isolated from cooked tenderizer A treated steak. Note areas of damaged sarcolemma marked with arrows. The striations were still present, though not apparent in the photograph. Thickness of these fiber preparations prevented simultaneous focus on all structures. Effect on the connective tissue was indicated by the very light staining with Van Giesson's. × 320.

Figure 9. A whole muscle fiber with attached disorganized muscle fiber envelope material isolated from cooked treated steak. × 320.

Figure 10. Parts of two whole muscle fibers, isolated from the same sample as used in the above figure, to show disintegration of the affected muscle fiber envelope into a fine granular state. × 320.

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STUDIES ON ENZYMATIC TENDERIZATION OF MEAT. III. HISTOLOGICAL AND PANEL ANALYSES OF ENZYME PREPARATIONS FROM THREE DISTINCT SOURCES[a]

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Increased interest has been shown in enzymatic tenderization of beef in reports from other laboratories *(1, 3, 4, 5, 7, 8, 10, 13)*. Correlations between morphological and panel observations of the effect of tenderizing agents have been conducted in our laboratories *(15, 18, 19)*. Several new enzyme preparations have since been investigated using additional histological techniques. This presentation contains findings directed toward these objectives: (a) to define and summarize the action of a total of twelve commercially available enzyme preparations on the structural components of muscle tissue, and to assess the activity of each of these preparations based on the extent of observed structural alterations; (b) to present panel data for those enzyme preparations that have been actually used for tenderizing freeze-dehydrated beef steaks; and (c) to evaluate the relationship of morphological to panel data thus obtained.

MATERIALS AND METHODS

Frozen-dried *longissimus dorsi* and *semitendinosus* steaks (mostly U. S. Utility grade) were used throughout this study; fresh beef was used only when it was desirable to obtain single muscle fibers for direct immersion in an enzyme solution.

Enzyme preparations represented 3 different origins or sources: (a) from either bacteria or fungi, protease 15, Rhozyme P-11 and A-4, HT proteolytic, fungal amylase, Hydralase D and TP; (b) from tropical plants, ficin, papain and bromelin; and (c) from pancreas, trypsin and Viokase. All of them were supplied to us by their manufacturer in powder form, some of them with known activity rating expressed in chemically assayed units. Concentration of enzyme preparation was expressed in per cent of the preparation in an aqueous solution. Except in elastic digestion, all experiments were carried out at room temperatures (approximately 21–25° C.) and no attempt was made to control the pH of the enzyme solution with buffers. In a few experiments, the final pH of aqueous enzyme solutions was determined.

A. Histological

The procedure of rehydrating frozen-dried meat in enzyme solutions *(16)* was used as the method for studying enzymatic action on muscle tissue. It has the advantage of quick uniform penetration of the enzyme. The standard procedure was to rehydrate strips of frozen-dried steak in an enzyme solution for 15 minutes. The treated material was fixed in 10% neutral formalin, celloidin sectioned at 10 μ, and triple-stained with hematoxylin, Van Gieson's and Weigert's.

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Direct immersion of *isolated* components in enzyme solutions allowed observation of maximal activity. The blendoring technique *(17)* was used for this purpose. Quantities of single muscle fibers were obtained from raw beef (Figure 2). After immersion in enzyme solutions, the fibers were washed, formalin-fixed, stained with hematoxylin and Van Gieson's, and mounted *in toto*. Treated fibers were measured for their muscle fiber extensibility *(17)*.

Collagenous and elastic fibers were isolated for separate study of enzymatic action, by dissection. Although perimysial sheets thus obtained contained both collagenous and elastic fibers, each tissue was studied individually in the following manner. The perimysial sheets were prepared for elastin study by staining with Weigert's overnight, and mounted *in toto*. Collagenous fibers in this material were not stained. A dry powder prepared from beef *ligamentum nuchae*, containing mainly elastic fibers, was used in several experiments. For collagen study the perimysial sheets were stained with Van Gieson's, and all elastic fibers left unstained. Results obtained this way were more clear-cut than if a sample had been stained for both types of connective tissue fibers. Control samples, not treated with any enzyme, were processed and stained in the same manner.

Enzymatic action on elastin digestion was observed under the compound microscope as it actually occurred. A piece of perimysial connective tissue, pre-stained with Weigert's, was mounted in a 5.0% ficin or papain solution on a slide, and covered with a coverslip. Progress of the enzymatic action on the elastic fibers was detectable within a few minutes and reached completion within half an hour. As digestion proceeded it was possible to observe the decrease in the intensity of the stain of the elastic fibers, the onset of segmentation of the individual fibers, and finally their complete dissolution. Photomicrographs were taken without disturbing the process under observation. When unstained elastic fibers *(ligamentum nuchae)* were used, photomicrographs were made using phase microscopy.

The structure of collagen fibers, unlike elastin, is affected by heat; hence it was necessary to subject enzyme-treated tissue to simulated cooking conditions. Small "pockets" were made with a scalpel in a frozen-dried steak and pieces of enzyme-treated perimysial sheet inserted in them. The steak was then rehydrated in water and broiled in the usual way. After cooking, the pieces were recovered, processed, and stained. This technique allowed study of the effect of high enzyme concentrations on collagen at elevated temperatures.

B. Panel analyses

The enzyme preparations chosen for taste panel study were Rhozyme P-11 and fungal amylase, both of fungal origin; ficin, papain and bromelin from plant sources. Selection of these enzymes was based upon the specificity and intensity of their activity demonstrated in the histologic studies. The concentrations of enzyme solutions used for rehydrating the steaks for panel study were arrived at by a series of screening tests comparing untreated and enzyme treated steaks. Enzyme levels sufficient to produce acceptable tenderness were established for both *longissimus dorsi* and *semitendinosus* steaks. The enzyme preparations were dissolved in either water or 2% sodium chloride solution for the studies made on *longissimus dorsi* steaks. *Semitendinosus* steaks were always rehydrated in 2% sodium chloride solutions. Loose tissue fragments, resulting from sawing frozen muscles into steaks before drying, were removed from the steak surfaces prior to rehydration. Steaks were left in the rehydrating solution for 30 minutes at room temperature, and cooked immediately. Steaks were cooked and prepared for taste panel analyses in the same way as described in a previous paper *(19)*. Amount of water taken up by the steaks during hydration and weight loss by evaporation and drip during cooking were recorded.

Adjoining steaks, one enzyme-treated and the other not treated, were compared directly for differences in tenderness. When more than one enzyme was being studied or when 2 enzymes were to be compared, consecutive steaks within one muscle were used and untreated steaks preceding and following the treated ones were analyzed as controls. Each treatment was repeated twice, using similar muscles from 3 animals. *Longissimus dorsi* steaks were given a single score for tenderness. *Semitendinosus* steaks were scored for both tenderness and the amount of residue remaining after a normal amount of chewing. Need for a further breakdown in panel scoring became apparent during the preliminary studies. A similar differentiation has been suggested recently by other

workers *(2)* who judged tenderness on the basis of softness, friability, and residual connective tissue. Adjoining steaks from *semitendinosus* muscle do not vary a great deal in tenderness or amount of residue *(6)*. Thus, scores given adjoining steaks may be compared directly.

RESULTS AND DISCUSSION

A. Histological

1. Muscle fiber structure. Degradation of the muscle fibers was a common property associated with the treatment with most of the proteolytic enzyme preparations used in these experiments. The mode of action on isolated fibers of Rhozyme P-11, and a commercial tenderizer containing papain *(19)*, were found to represent fairly well the general pattern.

The sarcolemma and the muscle fiber envelope were attacked first and disintegrated. Next, the nuclei of the muscle fibers, as well as those of the free cells of the endomysia, faded away or completely disappeared. The ensuing action of the enzymes progressively reduced the actomyosin complex to a state in which there no longer were visible cross-striations. The last stage involved the actual dissolution of the actomyosin. An over-all representation of the relative activities of the enzyme preparations, based on the degree of structural degradation, may be given in this order of increasing enzyme activity: untreated (Figure 2), bromelin (Figure 3), papain (Figure 4), *cf. (10)*, Hydralase TP (Figure 5), Hydralase D (Figure 6), fungal amylase (Figure 7), Viokase (Figure 8), protease 15 (Figure 9), and ficin (Figure 10) or HT proteolytic (Figure 11). Conditions of the nuclei, sarcolemma and above all, the state of the protein (actomyosin) were taken into consideration in this evaluation. Several exceptions to the general pattern of action were observed. Viokase (Figure 8) did not initially affect the sarcolemma and the muscle fiber envelope. Figure 7 shows an atypical well circumscribed oval-shaped area, in which the actomyosin was being dissolved. The remainder of the fiber was typically damaged by amylase.

2. Muscle fiber extensibility. Muscle fiber extensibility has proved to be inversely related to tenderness for beef muscle tissue from some groups of animals *(17)*; therefore, it is important to know how enzymatic action alters this property. This effect could not be demonstrated when control raw fibers possessed only slight initial extensibility. Control fibers with considerable initial extensibility were required, and the range of enzyme activity limited, in order not to destroy all the extensibility. Significant results were obtained by using fibers isolated from cooked *longissimus dorsi* which had 100% (or more) initial extensibility (i.e. were extensible to 10 mm. from an initial 5 mm. length), and by employing dilute enzyme solutions of 0.15% for one minute (Table 1).

These data proved that enzymatic action reduced muscle fiber extensibility and also suggested a probable correlation between the degree of extensibility loss and structural degradation. A relationship between sarcolemma destruction (with no loss of extensibility) and an increase in tenderness was demonstrated in the studies made with commercial tenderizers *(19)*. In the present study of cooked fibers, there was a loss of extensibility as well as destruction of the muscle fiber envelope and sarcolemma. It is possible that extensibility is a function of only muscle fiber substance condition, not sarcolemma, and that this in itself may be related to tenderness. This hypothesis is currently

under investigation. Two previous studies on the elongation of intact, undenatured fibers versus elongation of empty lengths of sarcolemma have been reported. Sichel *(12)* concluded that the property of elongation resides principally in the muscle fiber substance. Conversely, Ramsey and Street *(11)* found elongation to be a property of the sarcolemma and remnants of adhering connective tissue.

Detailed structural composition of the sarcolemma and muscle fiber envelopes, and their relationship to the specialized protein complex, actomyosin, were recently discussed *(14)*. Importance of these structural components for tenderness considerations resides in the fact that they are not destroyed by either the process of aging or cooking as is clearly illustrated in Figures 12, 13, and 14.

TABLE 1

Extensibility of cooked single muscle fibers immersed for one minute in 0.15% enzyme solutions

Enzyme preparations	Structural degradation as illustrated	Muscle fiber extensibility	
		Length over intial 5 mm.	Per cent loss
Control	Fig. 2	5.7	....
Bromelin	3	3.3	41
Papain	4	2.5	56
Hydralase TP	5	2.0	65
Hydralase D	6	1.6	72
Fungal amylase	7	1.2	79
Viokase	8	1.0	82
Protease 15	9	0.9	84
Ficin	10	0.0	100
HT proteolytic	11	0.0	100

3. Collagen. Two criteria were used for studying enzymatic action on collagen: (a) staining reaction of collagenous fibers with the acid fuchsin of Van Gieson's; and (b) structural alterations.

The mode of collagen degradation could be more advantageously studied in isolated perimysial sheets than in whole tissue. Figure 15 shows normal raw bundles of collagenous fibers, deeply stained with acid fuchsin. The fibers appeared dense, wavy and embedded in ground substance. The following sequence of changes (Figure 16) was typical of the action of ficin, bromelin, or papain: (a) a liberation of the ground substance; (b) a decrease in staining capacity; (c) a loss of both staining capacity and fibrillar character; and (d) reduction to an amorphous material.

Enzyme preparations derived from the tropical plants and pancreas were shown to possess collagenase activity. The microbial enzymes used had little or no such activity *(18)*. The action of four of the latter enzymes (HT proteolytic, Hydralase D, Hydralase TP, and fungal amylase), appeared to be localized on the ground substance or matrix *(9)* without affecting either the structure of the fibers or their affiinity for acid fuchsin.

Visible changes may be employed to measure the collagenase activity of any enzyme preparation. To illustrate, results of immersing comparable samples of perimysial material in 4.0% solutions of the 3 tropical plant enzymes are presented in Figures 17 (bromelin), 18 (ficin), and 19 (papain).

Note that the fibers treated with bromelin and ficin had almost reached the final stage of collagenolysis (i.e., comparable to d, Figure 16), whereas the fibers in papain presented a stage intermediate to stages b and c.

Collagen degradation by heat (Figure 20) and from immersion in 2.5% bromelin for 30 minutes (Figure 21) was roughly the same (from a structural standpoint).

Effect of combined enzyme action and subsequent cooking was noticeably greater than that of either alone. This can be appreciated by examining Figure 22, and comparing it with the two preceding figures. In line with this observation, collagen degradation seen in celloidin sections of cooked enzyme-treated steaks, was consistently more intensive and extensive than in cooked untreated steaks. A full description of Figure 22, illustrating this, follows. The material shown represents recovered masses of gelatinized collagen, resulting from isolated perimysial strands first immersed in 2.5% bromelin for 30 minutes, then implanted into a frozen-dried steak which was rehydrated in water and cooked in the usual manner. Degradation proceeded to stage d, Figure 16. When collagen fibers from cooked meat (Figure 23) were treated with enzyme solution (Figure 24), the degradation was much less (comparable to stage b, Figure 16).

Necessity for consideration of time and concentration of enzyme application is implied by these observations.

4. Elastin. Elastic fibers are intimately associated with collagen constituting a strong fibrous network throughout the muscle. They are present in the perimysia of tender muscles such as *longissimus dorsi* (Figure 25) and in greater numbers, and as larger fibers, in less tender muscles such as *semitendinosus* (Figure 26). These factors, in addition to its resistance to hydrolysis by heat, make elastin of importance in the evaluation of enzymes as meat tenderizing agents.

Enzymatic action on elastin was more discrete than that described for collagen, and was observed easily in fibers pre-stained with Weigert's. It began with a random segmentation of the individual fibers. Very fine fibers appeared as dotted lines (Figure 27); larger fibers (Figures 28 and 29) appeared as ribbons with clear unstained areas. These clear areas represented portions of the fibers that had been chemically altered so that they no longer took the Weigert's stain. The extent of this segmentation process increased with time until finally the body of the fiber appeared completely disintegrated (Figure 30). Careful inspection revealed "ghosts" of the original elastic fibers (see diagonal lines running from upper right to lower corner). Segmentation occurred regardless of whether the fibers were enzyme-treated first then stained (Figure 27), stained and subjected to the process of digestion (Figure 29), or not stained at all (Figure 31). In the latter case, phase contrast photomicrography was necessary. The similarity in the morphology of the enzyme-attacked elastic fibers obtained in all these different kinds of preparations, ruled out any possibility that the observed phenomenon might have been an artifact. Of the 3 plant enzymes, ficin ranked first (Figures 32 and 33), then papain (Figure 34), and finally bromelin (Figure 35) in elastolytic activity. The increased extent of elastin breakdown in Figure 33 over that shown in Figure 32 was due to a 20-minute difference in duration of treatment.

The microbial and fungal enzymes did not exhibit elastase activity. The only other preparations found to possess activity were trypsin and Viokase, both of pancreatic origin. Mode of attack on the elastic fibers was the same for all preparations, and observed differences in the degree of activity probably reflect the amount of elastase *(8)* present in each.

B. Panel Evaluation.

The importance of using more than one criterion for expressing tenderness is apparent from the data in Table 2. Bromelin, ficin or papain treatment resulted in an increase in initial tenderness and a decrease in amount of residue. Fungal amylase and Rhozyme P-11 treatment increased the initial tenderness of the meat but had very little effect upon residue except for elevated levels of Rhozyme P-11. The 2 lots of ficin studied were similar in their effect upon initial tenderness but ficin B was more active in reducing the residue. There was very little difference between the effect of 2 levels of ficin (0.0003 and 0.0004%) upon initial tenderness. The difference in the amount of residue was quite pronounced.

Differences in type of tenderizing action by the enzyme preparations are of special interest when related to their action upon the structural components of the muscle. The histological studies show the effect of much greater concentrations of the enzymes than were optimal for use in the panel studies. Later histological examination of tissues from the steaks cooked for panel evaluation indicated similar changes to a lesser degree. All of the enzymes brought about disintegration of the sarcolemma and muscle fiber envelopes and an increase in the initial impression of tenderness in the steaks. Only

TABLE 2

Increase in tenderness and residue scores due to enzyme treatment of semitendinosus steaks

Enzyme	Concentration %	No. of animals	No. of scores	Panel mean score [1]	
				Tenderness	Residue
Fungal amylase	0.000	3	24	6.5	5.5
	0.045	3	24	7.4	5.4
Rhozyme P-11	0.000	6	46	5.5	5.6
	0.010	6	46	6.4	5.4
	0.000	3	24	6.1	6.1
	0.015	3	24	7.2	5.0
	0.020	3	24	7.6	5.3
Bromelin	0.0000	3	24	6.5	5.5
	0.0003	3	24	7.6	4.5
Ficin A	0.0000	2	16	6.2	6.1
	0.0003	2	16	6.9	5.9
	0.0004	2	16	6.8	5.0
Ficin B	0.0000	3	24	5.8	5.8
	0.0003	3	24	6.5	5.2
	0.0004	3	24	6.8	4.4
Papain	0.0000	6	46	5.5	5.6
	0.0003	6	46	7.0	4.6

[1] Increasing tenderness 1-10. Increasing residue 1-10.

the plant enzymes—bromelin, ficin, and papain—exhibited collagenous activity in the histologic studies and produced a decrease in amount of residual tissue in the panel studies. These results suggest a direct relationship between these tissue changes and panel responses.

Presence of 2% sodium chloride in the rehydrating media provided an appreciable increase in tenderness of the *longissimus dorsi* steaks (Figure 1). The steaks, rehydrated in water and 2% sodium chloride, were taken from locations ½ inch apart in the rib eye. Addition of enzyme increased the tenderness scores of steaks rehydrated in either medium. The additive effect of salt and enzyme was a soft, rather mushy steak, indicating over-tenderization and an undesirable product. The optimal enzyme levels were different for steaks from the 2 muscles. The *semitendinosus* steaks required a larger amount of enzyme (Table 2 and Figure 1), even though their greater water absorption (Table 4) presumably produced a higher concentration of enzyme per steak.

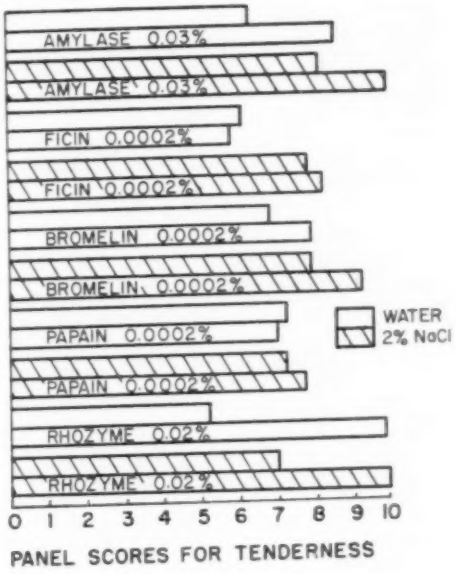


Figure 1. Tenderness scores for steaks rehydrated in water and 2% sodium chloride solutions, with and without enzyme added.

Enzyme preparations used were not comparable activity-wise on an equal weight basis. Hemoglobin and gelatin assay methods of expressing enzyme activity may not reflect the meat tenderizing properties of the enzymes used in this study. Whereas rhozyme and papain were fairly similar in their ability to hydrolyze gelatin (Table 3), about 150 times as much rhozyme as papain was needed to affect significantly the initial tenderness of meat (Table 2). Even at this concentration rhozyme produced much less change than did papain in the amount of residue remaining after chewing. It is interesting to observe, in this respect, the close relationship between changes in histological structure and panel response to tenderness differences *(18)*.

Rhozyme and papain at 4% concentration caused similar structural changes in actomyosin. Papain also affected the connective tissues, whereas rhozyme did not. The current gelatin and hemoglobin assays do not appear to reflect these enzyme properties.

Data obtained on the amount of water taken up by the steaks during rehydration and lost during cooking are summarized in Table 4. The *longissimus dorsi* steaks rehydrated in 2% sodium chloride solution absorbed more water during rehydration and lost less on cooking than did those rehydrated in water. The *semitendinosus* steaks represented two dehydration lots. The

TABLE 3

Enzyme activity as measured by hemoglobin and gelatin assay [1]

Enzyme	Hemoglobin Assay		Gelatin Assay	
	Hemoglobin units	Potency relative to ref. std.	Gelatin units	Potency relative to ref. std.
Reference				
Standard papain	5,300	1.0	1,300	1.0
Fungal amylase	1,900	0.4	1,500	1.2
Rhozyme P-11	2,900	0.6	6,700	5.4
Bromelin	14,800	2.8	18,200	14.5
Ficin	28,500	5.4	12,700	10.1
Papain	9,800	1.9	7,200	5.8

[1] These data were provided by Dr. Vincent Bavisotto of Paul-Lewis Laboratories, Inc., Milwaukee 12, Wisconsin.

TABLE 4

Weight changes during hydration in water and 2% sodium chloride solution and subsequent cooking

Rehydrating medium	Number of steaks	Average steak weight gs.	Weight-change gs./100gs. dry weight		Retention of resorbed water	
			Rehydration gain	Cooking loss	gs./100gs.	% of total
Longissimus dorsi						
Dehydration Lot No. 1						
water	10	32.9	157.0	56.2	100.8	64.2
water + enzyme	10	34.0	153.8	55.6	98.1	63.8
2% NaCl	10	35.1	169.4	51.0	118.4	69.9
2% NaCl + enzyme	10	36.1	166.4	48.6	117.8	70.8
Semitendinosus						
Dehydration Lot No. 2						
2% NaCl	21	13.5	276.4	83.6	192.8	69.8
2% NaCl + enzyme	40	13.5	279.3	82.3	196.9	70.5
Dehydration Lot No. 3						
2% NaCl	82	19.6	235.2	68.2	167.0	71.0

steaks in Lot Number 3 were larger in circumference than those in Lot Number 2. They picked up less water during rehydration but lost the same percentage of the resorbed water during cooking as did the other *semitendinosus* steaks and *longissimus dorsi* steaks. The amount of water present in the cooked steaks was a constant percentage of the amount absorbed during the rehydration period.

SUMMARY

A close relationship was demonstrated between enzyme-induced changes in tissue structure and panel response to tenderness differences. The ability of an enzyme to hydrolyze hemoglobin and gelatin did not reflect its activity as a meat tenderizer. A differentiation was made by the panel between initial tenderness and residue after chewing.

a) The initial tenderness was interpreted as being associated with disintegration of the sarcolemma and muscle fiber envelopes, and with reduced muscle fiber extensibility;

b) Residue was interpreted as being associated with degradation of collagen and elastin fibers. Enzymes of plant origin (ficin, papain, and bromelin) affected both of these groups of structures and properties. Microbial and fungal enzymes (Rhozyme P-11 and fungal amylase) affected principally the former group. Histological data alone were obtained for other microbial enzymes and the pancreatic enzymes Viokase and trypsin.

Larger quantities of enzyme were needed to produce tenderness in steaks from *semitendinosus* than from *longissimus dorsi* muscles. In the latter steaks the presence of 2% sodium chloride in the rehydrating media produced a marked increase in tenderness.

Explanation of Figures

Figures 3 through 35 present structural alterations observed during these studies on enzymatic tenderization of meat. Figures 12, 13, 14, 25, 26, 32, 33, 34, and 35 are 10-micron celloidin sections triple-stained with hematoxylin, Van Gieson's and Weigert's. The remainder of the figures are whole mounts: of these, tissues in Figures 2 through 11 are stained with hematoxylin and Van Gieson's; tissues in Figures 15 to 24 inclusive, with Van Gieson's only; and tissues in Figures 25 to 30 inclusive, with Weigert's only.

Figure 2. Intact muscle fibers isolated from raw *longissimus dorsi*. ×320.

Figures 3–11. Comparison of morphology of muscle fibers after 1 minute direct immersion in 4.0% aqueous solution of various enzyme preparations:

Figure 4. Papain.
Figure 5. Hydralase TP.
Figure 6. Hydralase D.
Figure 7. Fungal amylase.
Figure 8. Viokase.
Figure 9. Protease 15.
Figure 10. Ficin.
Figure 11. HT proteolytic. All figures ×320

Figure 12. X-section of 28-day aged *longissimus dorsi* showing intact sarcolemma of autolyzed muscle fibers. ×650.

Figure 13. L-section of cooked fresh *longissimus dorsi* showing intact sarcolemma. ×1400.

Figure 14. L-section of cooked 28-day *longissimus dorsi* showing intact sarcolemma. ×150.

Figures 15-24. Enzymatic degradation of collagen in isolated perimysial connective tissue.

Figure 15. Normal bundles of collagenous fibers (raw control), stained deep red with Van Gieson's.

Figure 16. Collagenous fibers dissected out from cooked *semitendinosus* steak and immersed in 2.5% papain for 30 minutes.

Figure 17. Collagenous fibers (raw) after immersion in 5.0% bromelin for 2 hours.

Figure 18. Collagenous fibers (raw) after immersion in 5.0% ficin for 2 hours.

Figure 19. Collagenous fibers (raw) after immersion in 5.0% papain for 2 hours.

Figure 20. Cooked collagenous fibers dissected out from a *semitendinosus* steak.

Figure 21. Collagenous fibers from a raw freeze-dehydrated steak after it was rehydrated in 2.5% bromelin for 30 minutes.

Figure 22. Hydrolyzed collagen dissected out from the above enzyme-treated steak after it was cooked.

Figure 23. Collagenous fibers dissected out from a cooked *semitendinosus* steak not treated with enzymes.

Figure 24. Similar cooked collagenous fibers as in above figure after immersion in 2.5% bromelin for 30 minutes. All figures ×320.

Figures 25-31. Enzymatic digestion of elastin.

Figure 25. Normal perimysial elastic fibers in raw beef *longissimus dorsi*. ×320.

Figure 26. Normal perimysial elastic fibers in raw beef *semitendinosus*. ×320.

Figure 27. Partially digested elastic fibers in isolated perimysial sheet treated with 1.5% Viokase for 16 hours. ×320.

Figure 28. Perimysial sheet treated with 0.05% ficin for 16 hours showing an intermediate stage of digestion of elastin on a massive scale. ×320.

Figure 29. Perimysial sheet previously stained with Weigert's after 4 hours direct immersion in 5.0% ficin, showing typical pattern of elastin digestion *in situ*. ×600.

Figure 30. Perimysial sheet showing completely digested elastic fibers after 60 minutes in 1.5% ficin. Note "ghosts" of elastic fibers running in the direction of the arrow and lying against a background of unstained collagenous fibers. ×320.

Figure 31. Similar material as in Figure 29 except the perimysial sheet was unstained and was photographed with phase contrast. ×1250.

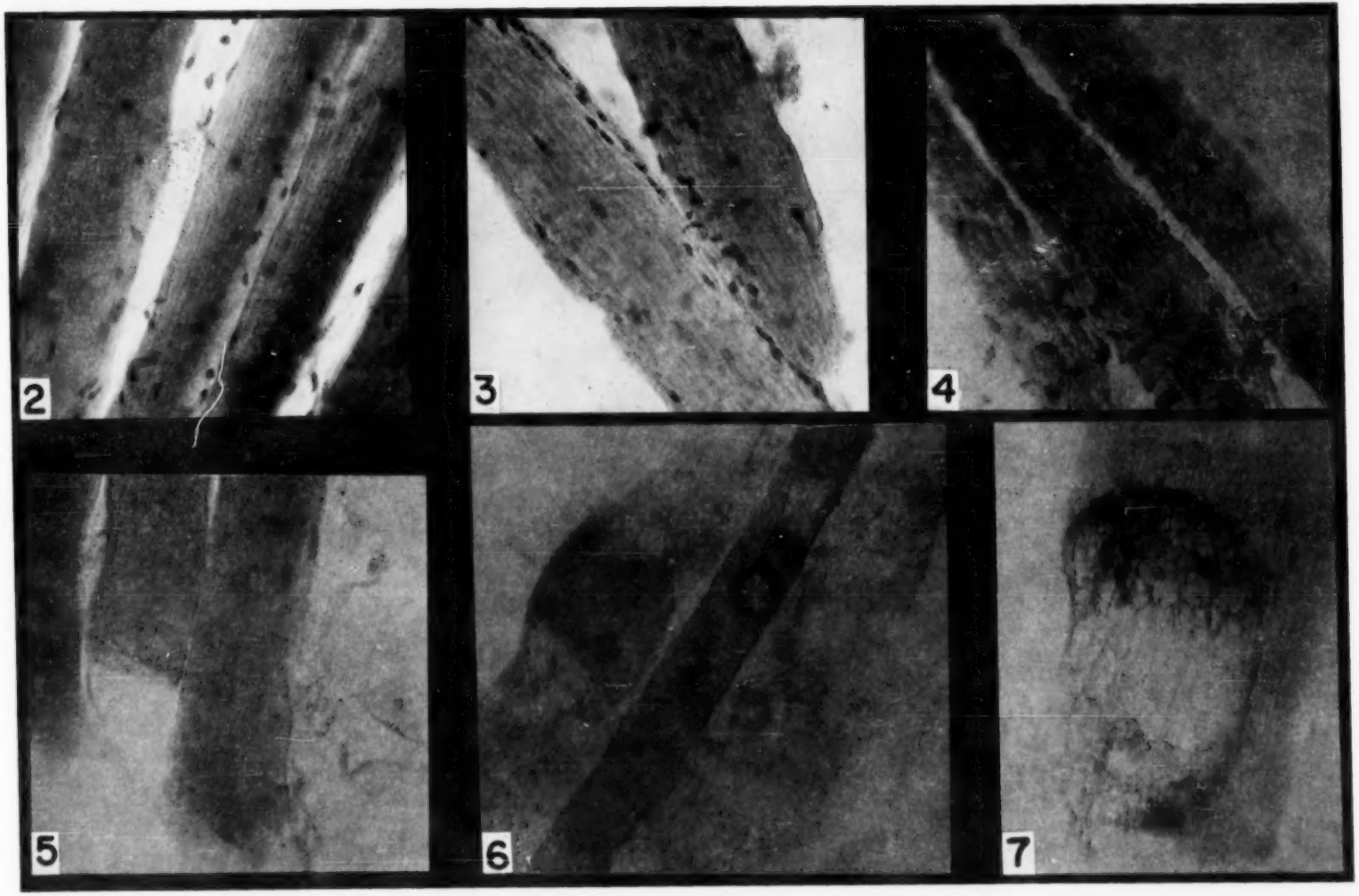
Figures 32-35. Pattern of elastin digestion seen in celloidin sections of small samples of freeze-dehydrated *semitendinosus* steaks rehydrated in 1.5% of different enzyme preparations:

Figure 32. Ficin, 40 minutes.

Figure 33. Ficin, 60 minutes.

Figure 34. Papain, 60 minutes.

Figure 35. Bromelin, 60 minutes. All figures ×320.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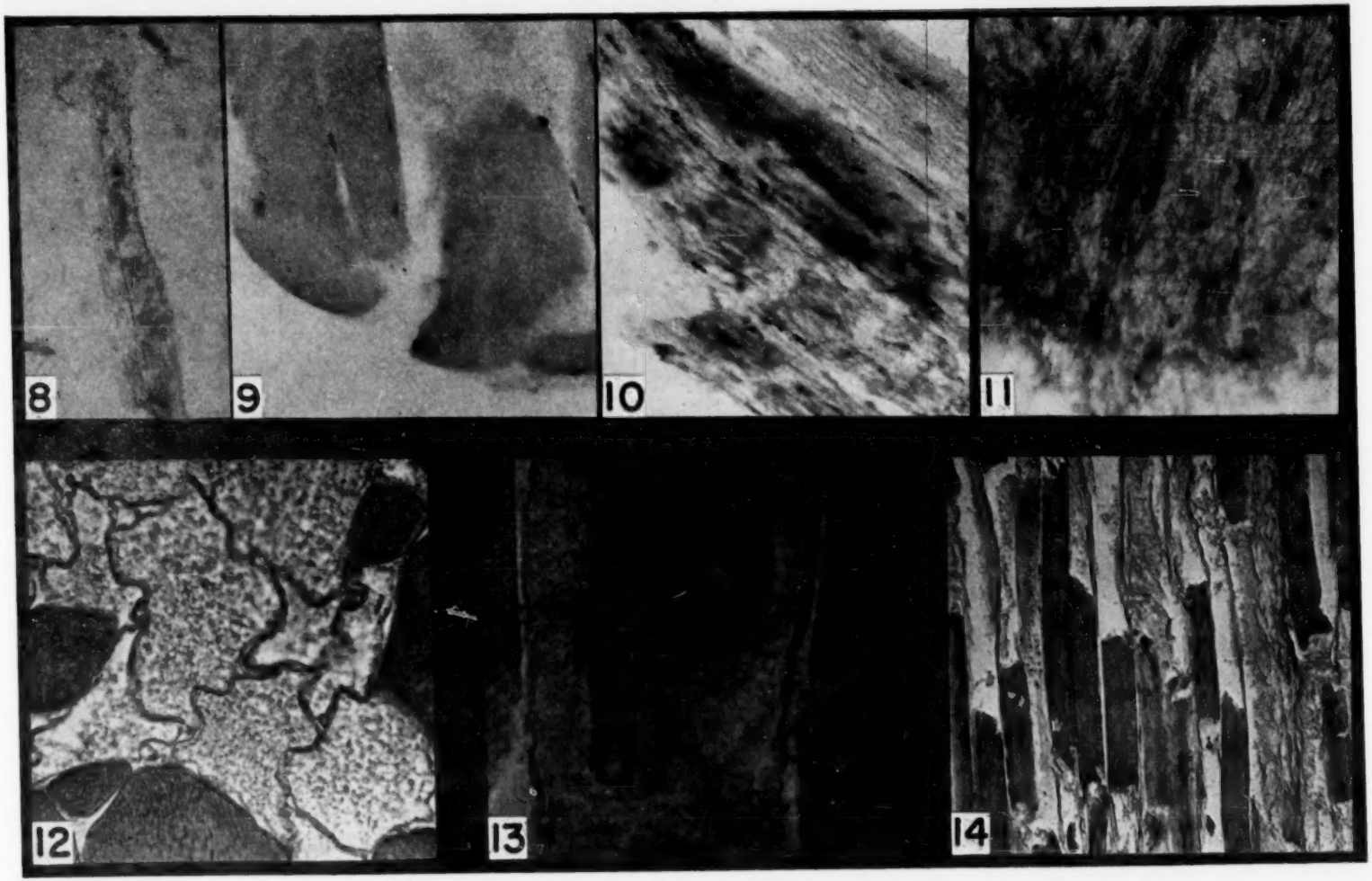
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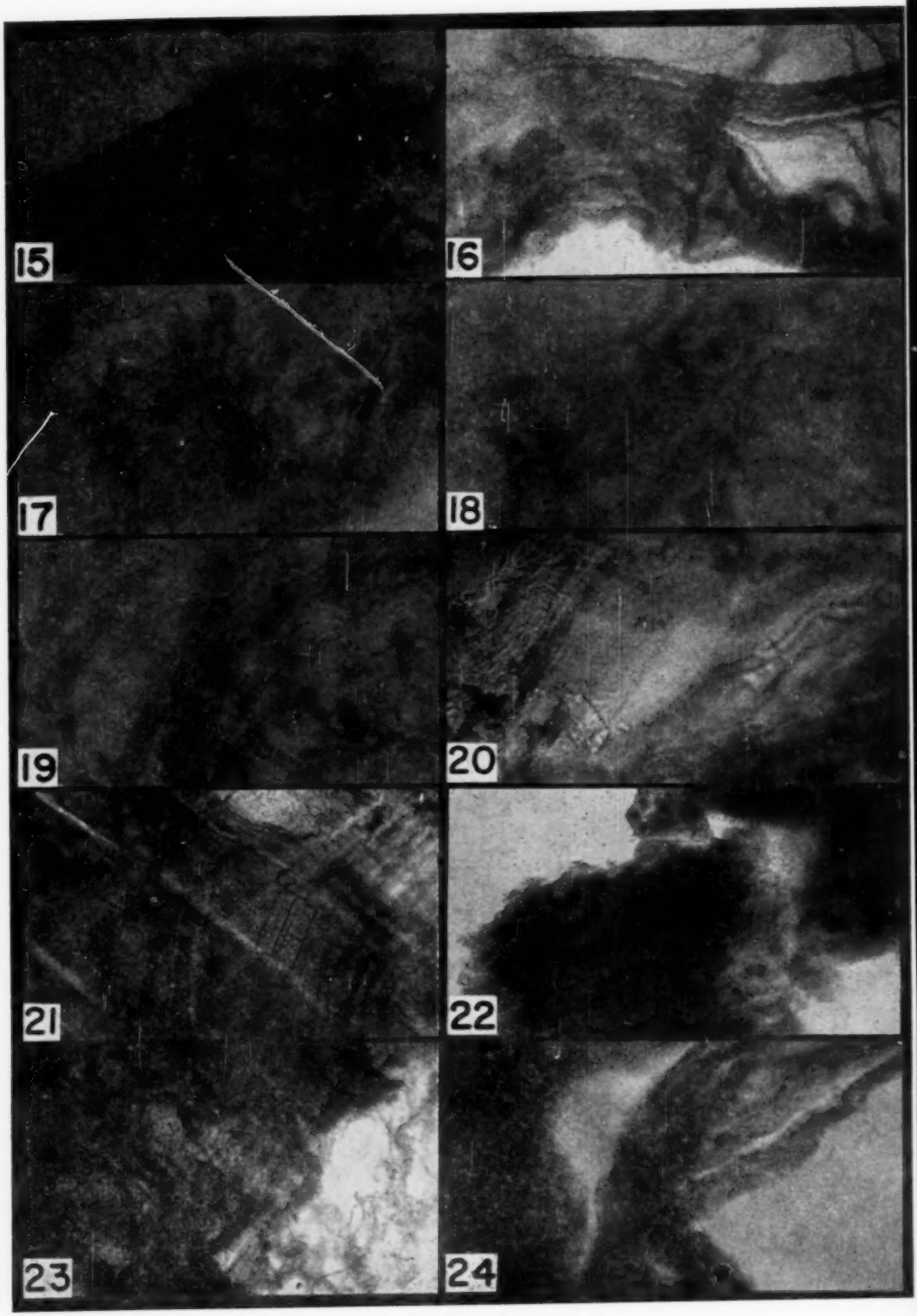
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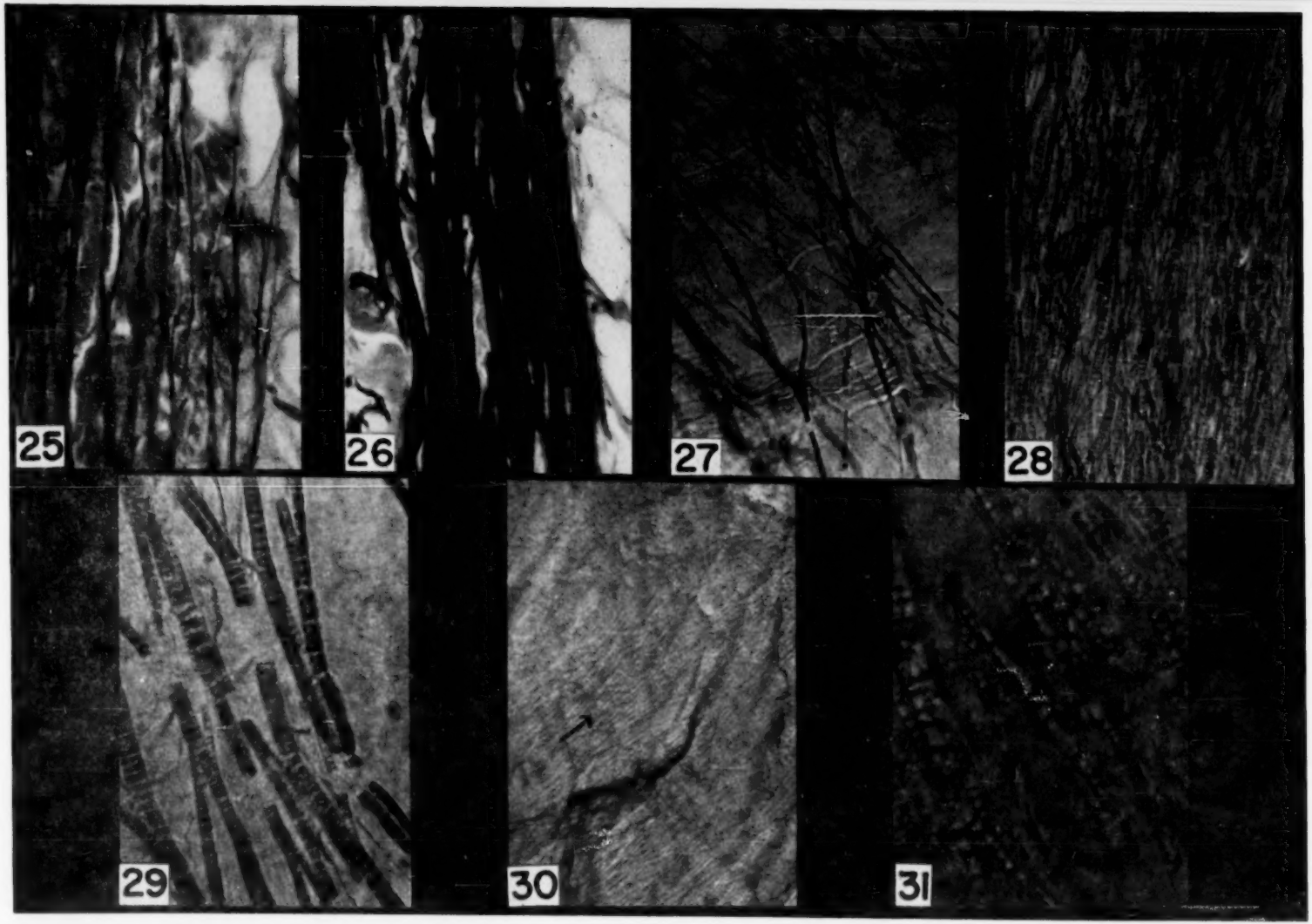
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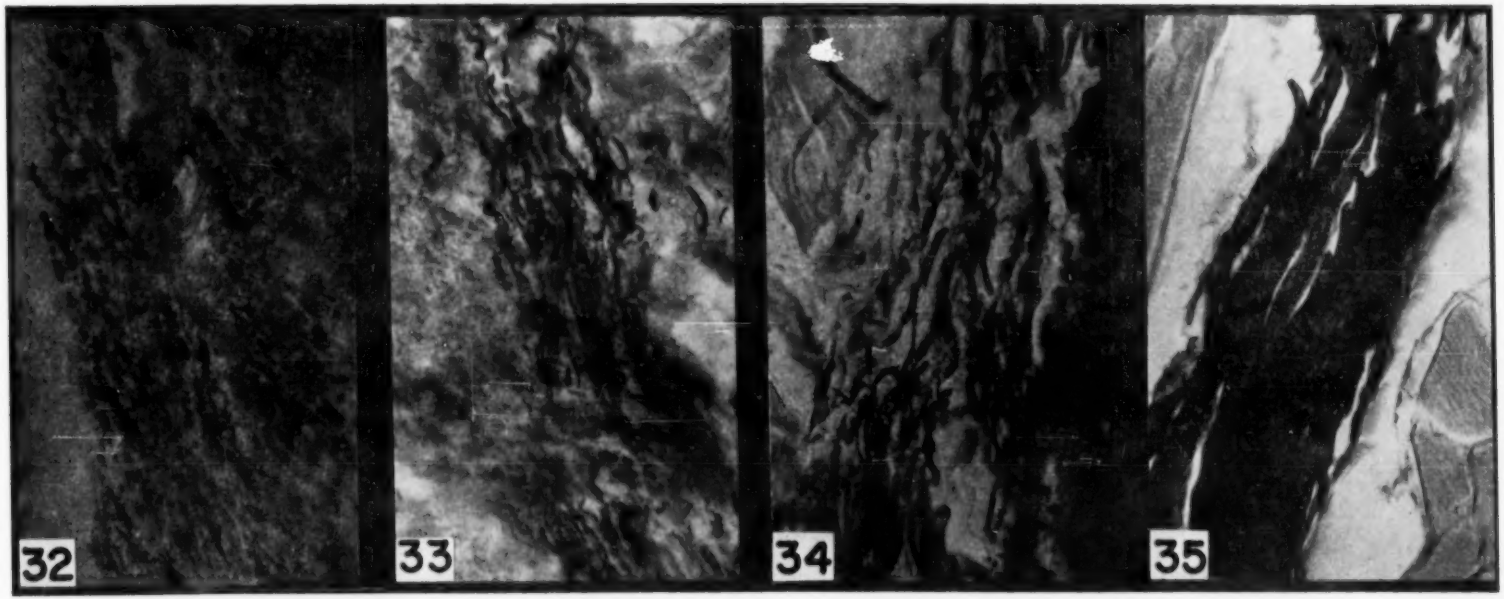
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Acknowledgment

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THE PREPARATION OF LIPASE FROM PEAS AND THE ACTION OF STEAPSIN AND PEA LIPASE ON CRUDE PEA LIPID[a]

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(Manuscript received April 3, 1958)

Lipase is known to be present in a number of seeds. Its presence in castor beans was noted some time ago *(3)*. This enzyme has been found only in small quantities in *Pisum arvense (1)*. Small amounts of lipase were found in germinating peas, but none in resting pea seeds *(4)*.

Previous work in this laboratory established the fact that crude lipids extracted from raw garden peas (Thomas Laxton variety) which had been held in frozen storage at — 17.8° C. for 39 days contained much more titratable acid than that extracted from the fresh peas previous to storage *(8)*. This suggests that an active lipase is present.

The present report covers the preparation of lipase from peas and shows that the bulk, if not all of this lipase is water-soluble.

MATERIALS AND METHODS

Part of the work recorded in this paper was done by employing fresh peas as the starting material. However, it was necessary to insure an available supply of raw material for use beyond the normal pea season. It had been demonstrated that peas dried by lyophilization in Stokes Freeze Drying Equipment to a moisture content of about 3% held their quality in storage at — 17.8° C. for reasonably long periods of time (Table 1). Furthermore, it was ascertained that lipase was not inactivated by this treatment.

TABLE 1
Acid and peroxide numbers of crude lipid extracted from frozen raw peas and lyophilized raw peas after storage at — 17.8° C. (Thomas Laxton variety)

Condition	Acid number		Peroxide number	
	Initial	After storage	Initial	After storage
Frozen raw peas held at — 17.8° C. for 11 months	16.4	82.4	0	4.9
Lyophilized raw peas held for 10 months	10.2	14.8	0	5.0

Lipase activity was measured by two methods. The first of these, a modification of that used by Martin and Peers *(10)* for oat lipase, is based on the liberation of fatty acids from olive oil, or from crude lipid extracted from blanched peas, as substrate, in a dough of controlled moisture content. The fatty acids are extracted from the dough with ethyl ether and titrated with standard alkali. The procedure used follows. One-half gram of blanched pea powder was used to form dough. This powder was obtained by ethyl ether ex-

[a] Approved by the Director of the New York State Agricultural Experiment Station as Journal Paper No. 1044.

traction of lyophilized frozen blanched peas, previously ground to 40 mesh. One hundred and fifty milligrams of lipid material was added and mixed thoroughly with the pea meal in a small Pyrex test tube. Two-tenths ml. of enzyme solution was then added and mixed to form a dough. The tube was stoppered and immersed in a constant temperature water bath at 37° C. After incubation for 24 hours the mixture was transferred with agitation to a 100 ml. centrifuge tube with 35 ml. of ethyl ether. The tube was stoppered with a rubber cap, and centrifuged at 1600 r.p.m. in an International No. 2 centrifuge for 15 minutes. The supernatant ethereal solution was then decanted into a 100 ml. extraction flask. The ether was removed by means of suction and the residual oil was titrated with 0.0354 N sodium hydroxide (1 ml. = 10 mg. free oleic acid). Since the oil recovery is nearly 100%, the results can be calculated on the basis of the weight of the olive oil or crude lipid originally added. The preparation of this crude lipid was described in a previous publication *(7)*.

The manometric procedure employing tributyrin as substrate as recommended by Martin and Peers *(10)* was also employed in this work. The readings, however, were taken at 5-minute intervals for ½ hour and then at 10 minute intervals thereafter during a 1 hour run. The tributyrin used was a 2 per cent (w/v) dispersion in 0.0148M $NaHCO_3$.

The determination of acid and peroxide numbers was described in previous publications *(5,7)*.

EXPERIMENTAL AND RESULTS

The activity of a commercial lipase preparation (steapsin, Nutritional Biochemicals Corp.) was checked in the modified dough method with varying amounts of the enzyme, and it was found that increasing quantities of acid were produced with the use of increasing amounts of enzyme.

Steapsin activity was determined using crude lipid extracted from blanched peas as the substrate in the modified dough method. The extracted pea meal was used as the carrier in this experiment. For comparative purposes, olive oil substrate was used in a companion series of tubes. The reaction mixtures were incubated as described under Materials and Methods. The results are given in Table 2.

TABLE 2

Activity of commercial lipase (steapsin) in modified dough method using blanched pea lipid substrate

Condition	Total acid as mg. of oleic acid	Acid number
Blanched pea lipid plus steapsin	6.71	80.9
Blanched pea lipid plus water (blank)	1.48	19.7
Olive oil plus steapsin	6.25	78.7
Olive oil plus water (blank)	0.20	2.6

From Table 2 it can be seen that blanched pea lipid can serve as a substrate in the dough method as effectively as olive oil. The blank in the case of pea lipid is somewhat higher. The acid numbers obtained in the lipase-treated samples approached those found in the lipid extracted from frozen raw peas after one year's storage at −17.8° C. This comparison may not be strictly valid because the lipid concentration in the dough method is about 30 times that normally present in peas.

Pea crude lipid contains chlorophyll *(6)*. A conversion of chlorophyll to pheophytin was observed visually in the doughs and ether extracts obtained from the doughs treated with steapsin. These findings of increased acid number and change of chlorophyll to pheophytin are in accord with the observation previously made on lipids ex-

tracted from frozen raw peas following prolonged storage at − 17.8° C. *(5, 6, 7, 12)*. To confirm the visual observation of pheophytin formation, the absorption spectrum of the extracted pigments was determined, by means of a Beckman DU spectrophotometer, using the method of Mackinney *(9)*. Results are shown in Table 3 and Figure 1.

TABLE 3

Destruction of chlorophyll by lipase (steapsin) *in vitro*

Conditions	Chlorophyll		
	mg./l.	mg./g.	% destroyed
36.7 mg. blanched pea lipid plus water	4.25	5.79	0
37.5 mg. blanched pea lipid plus steapsin	2.45	3.27	43.5
22.3 mg. blanched pea lipid plus steapsin	1.73	3.88	33.0

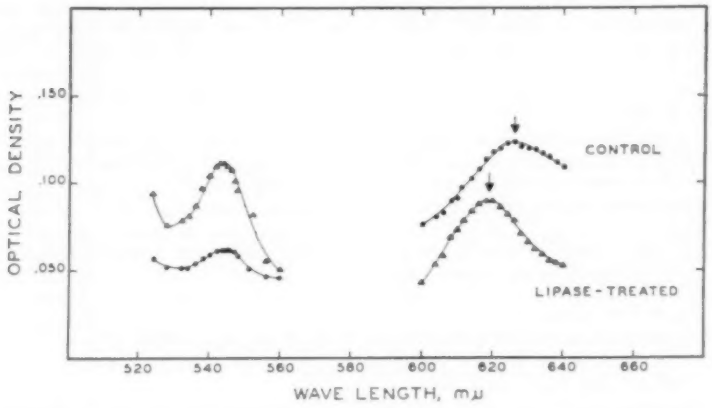

Figure 1. Changes in absorption spectra produced by lipase acting on blanched pea lipid.

Figure 1 shows a marked shift in the absorption peaks in the neighborhood of 620 mμ and a large increase in the peak at 545 mμ in the spectrum of pigments from lipase-treated doughs. Both phenomena are characteristic when chlorophyll changes to pheophytin. This destruction of chlorophyll occurred as a result of lipase action, and was not caused by lipoxidase, for the steapsin used was devoid of lipoxidase activity.

A comparison of the dough method with the manometric method for lipase determination showed the latter to be less time consuming and more precise, enabling the detection of smaller quantities of lipase. One mg. of steapsin acting on 16.5 micromols of tributyrin gave 15.5 micromols of CO_2, or 94% of the theoretical. On this basis, the method seemed applicable to the problem at hand.

Pea lipase. Manometric determinations on fresh peas showed that lipase is present. One-half gram of fresh peas gave 20.7 micromols of carbon dioxide when acting on 33.1 micromols of tributyrin.

Lipase was extracted from lyophilized peas by the following process.

Lyophilized peas were rehydrated in closed vessels at 2° C. over water to a moisture content of 11%. They were then ground to pass a 40-mesh screen in a micro Wiley mill, and extracted for 6 hours with ethyl ether in Soxhlet extractors. The Soxhlet-extracted meal was freed from ether by spreading it in a thin layer on watch glasses at room temperature in the hood. Twenty grams of meal was sifted into 180 ml. of cold N/40 NH_4OH *(2)* contained in a Waring blender cup and agitated carefully to effect thorough wetting and distribution. The mixture was allowed to stand in the cold room (2° C.) for ½ hour, and was then blended for 2 minutes. It was then poured into a conical glass percolator with a glass wool plug in the bottom. The dropwise flow rate was controlled by means of a rubber tube and screw clamp. When percolation was nearly complete, a second charge of 100 ml. of cold N/40 NH_4OH was added and allowed to percolate through the pea meal. These operations were carried out in the cold room at 2° C. The resulting extract was centrifuged for 30 minutes at 17000 × g in the Servall vacuum centrifuge. The supernatant was dialyzed against cold running tap water at 7° C. to 9° C. for 24 hours. The precipitate which formed during dialysis was removed by again centrifuging the dialyzed material for 30 minutes at 17000 × g. The final supernatant liquid was then lyophilized, weighed, and stored at − 17.8° C. The yield was 1.45 g. of lyophilized lipase preparation from 20 g. of starting material.

The lipase activity of all fractions was measured by means of the manometric assay method. Data are given in Table 4.

Precipitates obtained in the two centrifugations mentioned last contained negligible amounts of lipase activity, and were discarded. The bulk of the lipase, if not all, appears to be in the soluble form. Another preparation was made as just described, except that after dialysis the precipitate formed during dialysis was removed by centrifuging at 100,000 × g. at 0° C. in a Spinco preparative ultracentrifuge. Results are shown in Table 5.

Data presented by Bamann, Ullman and Tietz *(2)* on soluble lipase preparations from seeds of *Nigella sativa*, *Helleborus niger*, and certain other plants was obtained by centrifuging at 4,000 r.p.m. for 20 minutes.

Present data on pea lipase resulted from centrifuging the preparation for one hour at 100,000 × g, and yielded clear supernates. This definitely established the fact that soluble lipase capable of hydrolyzing tributyrin is present in peas.

Little activity was found in the precipitates obtained by centrifugation at 100,000 × g.

The activity of the lyophilized pea lipase preparations was compared to those of commercial lipase (steapsin) using the manometric assay method with tributyrin as substrate. On a dry weight basis, the steapsin was 400 times as active as the lipase prepared from peas. Fresh peas gave 0.022 micromols of carbon dioxide per milligram of material. Pea lipase gave 0.65 micromols per milligram. This shows a concentration of lipase activity against tributyrin of approximately 29 times based on the fresh weight of peas.

When tested in the Warburg apparatus, pea lipase was shown to hydrolyze 2% tributyrin, but not olive oil or crude pea lipid.

An experiment was set up in which a slurry of blanched peas was used in place of the olive oil or crude pea lipid as substrate. Since the blanched peas contain the natural substrates for pea lipase but no active enzymes, activity should be obtained on the addition of the pea lipase, under suitable experimental conditions. Prepared pea lipase and steapsin were tested separately in this manner.

Steapsin, 1 mg., gave 5.5 micromols of carbon dioxide. Pea lipase, 15 mg., gave 0.03 micromols of carbon dioxide under the same conditions. These figures are net values; the controls having been subtracted. It appears, therefore, that the steapsin is 183 times as active as the prepared pea lipase when acting on the lipid matter in peas.

DISCUSSION

Large increases in titratable acidity in lipids extracted from frozen raw peas following prolonged storage may best be explained as having resulted from the action of lipase. That lipase can function at low temperatures has been known for some time *(12)*. Addition of pancreatic lipase to macerates of enzymatically inert blanched peas resulted in the production of large

TABLE 4

Preparation of pea lipase and activity of the fractions

Step No.	Fraction	Volume or weight	Volume or weight used for analysis	CO_2 release in micromols	Total solids in sample gm.	Total micromols of CO_2 from original volume	Micromols of CO_2 from 1 gm. of dry weight
1	Original slurry (20 g. + 80 ml. H_2O)	100 gm.	1 gm.	28.2	0.1825	1820	151.5
2	Ether extracted (20 g. + 80 ml. H_2O)	100 gm.	1 gm.	21.4	0.1826	2140	117.2
3	Crude extract from percolator	218 ml.	1 ml.	9.6	0.0385	2093	249.3
4	Supernatant from centrifugation of crude extract at 17000 × g	196 ml.	1 ml.	9.0	0.0362	1764	248.6
5	Supernatant from dialyzed and centrifuged (17000 × g) extract	209 g.	1 ml.	5.5	0.0076	1150	723.2
6	Lyophilized step 5	a) 0.0076 g. to 1 ml. b) 0.0152 g. to 1 ml.		4.2 9.9			

TABLE 5
Effect of centrifugation on activity of pea lipase

Centrifugation of extract	Substrate	Micromols of CO_2 released [1]
17,000 × g	Tributyrin	11.9
17,000 × g	Blanched pea slurry	2.9
100,000 × g	Tributyrin	8.3
100,000 × g	Blanched pea slurry	0.5

[1] 30 mg. of lyophilized pea lipase preparation per flask.

quantities of acid after 17 months of storage at —17.8° C. *(11)*. The onset of off-flavor during frozen storage of raw peas closely paralleled the increase in acid number of the crude pea lipids *(8)*. It has recently been proposed that lipase may function as an adjunct to lipoxidase in off-flavor development in frozen raw vegetables through a labilization of fatty acid substrates *(11)*.

Data obtained indicate that lipase prepared from peas is active mainly in splitting tributyrin but also showed slight action in splitting the lipid in peas. This was found to be true whether the enzyme was extracted from fresh peas, or from fresh peas which had been dried by lyophilization, and stored at — 17.8° C. until used. This latter was found to be satisfactory as a means of storing the vegetables for use in the preparation of enzymes over extended periods of time.

The method used for the extraction of lipase from peas involved the use of N/40 NH_4OH as the solvent. This was recommended by Bamann, Ullman, and Tietz *(2)*, who also employed water and aqueous glycerol as solvents for plant lipase. In the present study it was found that the weak ammonium hydroxide was the most satisfactory of the solvents recommended.

In the preparation of this enzyme, the centrifugation at 100,000 x g seems to permit the conclusion that the bulk if not all of the lipase of peas is soluble.

Ammonium sulfate fractionation was found to be unsatisfactory as a method of concentration because of the large losses of enzyme in the process. Likewise, fractionation with cold acetone was found to result in losses too large for practicability.

The pea lipase preparation appears to be reasonably stable in the dry form at — 17.8° C.

SUMMARY

A study has been made of the action of steapsin and pea lipase on crude pea lipid. A lipase (steapsin) was shown to be effective in changing chlorophyll to pheophytin. Manometric techniques showed that steapsin was 183 times the strength of prepared pea lipase based on activity on pea lipid, and 400 times the strength of prepared pea lipase based on activity on tributyrin. Pea lipase was concentrated 29-fold compared with the activity of fresh peas. Ammonium sulfate and cold acetone were unsatisfactory as a means of concentration of pea lipase. Much of the lipase of peas is soluble in water. The pea lipase was shown to have slight activity on blanched pea slurry, using the manometric technique.

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DIASTASE ACTIVITY AND HYDROXY-METHYL-FURFURAL IN HONEY AND THEIR USEFULNESS IN DETECTING HEAT ALTERATION

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(Manuscript received March 3, 1958)

Honeys are considered adulterated in Europe if they do not meet the requirements of the continental honey regulations regarding diastase activity and hydroxy-methyl-furfural (HMF) content. A very low diastase activity allegedly indicates that the honey has been subjected to unfavorable temperature conditions *(6)*, since diastase activity of natural honey is rapidly reduced when honey is heated *(1, 2, 6, 7, 8)* or stored at unfavorable temperatures. Similarly, the presence of an appreciable amount of HMF has been interpreted as evidence of alteration of honey by heat. Gothe's suggestion that the Fiehe test for HMF should be used in conjunction with the diastase assay in judging a honey of low diastase has evidently become a rule in the control laboratories of Germany *(4)*.

According to the German honey regulations, any alteration or change in the composition of natural honey is to be considered an adulteration. Heating honey to a very high temperature is believed to destroy certain dietetic values of honey *(7)*. Natural honey has been shown to have a certain bactericidal substance, often called inhibine, which is heat labile *(3, 9, 10, 11)*. Honeys, therefore, may show less bactericidal action after being heated. At present, the sensitivity of the "inhibine" of honey to heat seems to be a major reason, although still not the only reason, for the unacceptability of heated honeys in Germany.

Many tests have been devised to determine the extent of the heat treatment used in preparing honey for trade and customer acceptance *(3, 5, 6, 8, 13)*. The diastase test and the HMF test are simpler, more rapid, and less expensive to make than the biological analysis for bactericidal quality. However, because of the large variation in diastase activity among honeys as well as the lack of a simple, objective method of assaying diastatic activity precisely, numerous doubts have arisen from time to time concerning the validity of the regulations of the German Food Control in regard to the exact amount of diastase that must be present for a honey to be considered unheated *(7)*. Similarly the qualitative test (Fiehe test) used to detect HMF in honey is of doubtful usefulness, because the results are subject to the discretion and/or experience of the analyst.

The present work involves the development of more precise and reproducible methods of measuring diastase activity and HMF, together with a study of the natural occurrence of these substances in honeys and the effect of heat on them.

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EXPERIMENTAL PROCEDURES

Samples. Samples of honeys were collected over a period of 2 years from producers and buyers in California. An attempt was made to secure unheated samples of known origin. Some samples were received in the comb; others had been drained from the comb without heat; and still others were obtained by procedures normally used for extraction by producers in California.

Heat treatments. Certain samples of honey were exposed to elevated temperatures for specific periods. These samples, in 7-dram screw-cap vials were brought to temperature over a 15-25 minute interval in an oven controlled at the desired temperature (in later work a water-bath was used) and held in incubator rooms or the water-bath for the specified time intervals. Heat treatment was stopped by allowing the samples to cool in air to room temperature.

Analytical procedures

(a) *Determination of diastase (alpha-amylase) activity:* A method of determining diastase activity in honey was developed by modifying the method used by Schwimmer *(12)* for measuring alpha-amylase activity of barley malt. The modified method employs a photoelectric colorimeter to measure the time required for a specified amount of honey to decompose a specified amount of starch to a prescribed endpoint. Under standard conditions the time to reach the endpoint is inversely proportional to the enzyme concentration (see Fig. 1). Although the basic principle of such a method is well established for determining alpha-amylase activity, the early methods involved visual determination of the endpoint, so that the results were not precise nor easily reproduced.

For the purpose of comparison a modification of the method of Gothe was used to measure the diastase activity of several honeys assayed with the previous photoelectric method. The modified method of Gothe employed was obtained from Dr. H. Duisberg, Leiter des Instituts fur Honigforschung at Bremen, Germany; the method is similar to that used by Kiermeier and Koberlein *(7)*.

Reagents

(a) *Stock solution of iodine.* Dissolve 11.0 g. of resublimed iodine in 30 to 40 ml. of a solution containing 22.0 g. of KI and dilute the resulting solution to 500 ml. with distilled water. This solution is sufficiently stable for 1 year when stored in a glass-stoppered, dark bottle.

(b) *Dilute iodine solution.* 0.0007 N. Dissolve 20 g. of KI and 2 ml. of the stock solution in distilled water and dilute to 500 ml. Prepare a fresh solution every few days.

(c) *Acetate buffer.* pH 5.3, 1.59 M. Dissolve 43.5 g. of CH_3 COO $Na \cdot 3H_2O$ and 4.5 ml. of glacial acetic acid in water and dilute to 250 ml.

(d) *Starch solution.* Weigh accurately 2.000 g. of soluble starch (e.g., Phanstiehl, reagent grade, improved Lintner method) into a 250 ml. Erlenmeyer flask. Add 90 ml. of water and mix by swirling. Bring the mixture to a boil rapidly by swirling the suspension over a Fischer burner; then boil it gently for 3 minutes. Immediately transfer the solution to a 100 ml. glass-stoppered volumetric flask and cool under tap water to room temperature. Dilute to volume and mix thoroughly. Prepare fresh every 2 days.

(e) *Sodium chloride.* 0.5 M. solution.

Preparation of sample

20% honey solution. Weigh accurately 10.00 g. of honey into a small beaker. Dissolve the sample in a small amount of water and 5 ml. of acetate buffer. Transfer this solution quantitatively to a 50 ml. volumetric flask containing 3 ml. of 0.5 M-NaCl solution. The NaCl must not be added before the honey is dissolved in buffer, since diastase activity is rapidly lost at pH values below 3.9 or 4.0 in the presence of NaCl.

Apparatus

(a) *Reaction vessel.* A special glass reaction vessel should be used to enable rapid mixing of the honey and starch solutions. This vessel consists of a glass-stopped tube with a side arm. It can be made from 17 mm. tubing with a 19/22 T joint and a 19 T solid stopper. With the tube in a vertical position the main compartment should hold about 13 ml. and the side compartment about 6 ml.

(b) *Water bath.* A thermostatically controlled water bath capable of maintaining a temperature of 40.0° C. ± 0.2° C.

(c) *Photoelectric colorimeter.* A Klett-Summerson photoelectric colorimeter, model no. 900-3, (Klett Manufacturing Co., 181 E. 87th Street, New York, N. Y., USA.) equipped with a No. 66 filter, or a similar instrument.

Determination

Standardization of reaction mixture. The dilution necessary to bring the zero-time sample within the range measurable with the colorimeter must be determined in advance. It is a constant value for a given starch.

Mix 5 ml. starch solution with 10 ml. distilled water. Take 0.5 ml. of this starch solution and add it to 5 ml. of the dilute iodine solution. Determine the amount of distilled water required to bring the color intensity of the mixture within a Klett reading of 850-900 or a transmittance of 1.7-1.8.

Digestion and color measurement procedures. Pipette 10 ml. of the 20% honey solution into the main compartment of the reaction vessel, and 5 ml. of starch solution into the side arm. Place the reaction vessel into the 40° C. water bath. Avoid any premature mixing of the reactants during these steps.

Allow 15 minutes for the contents of the flasks to warm up to bath temperature, then start the digestion by tipping the reaction vessels to cause the contents of the side arm to mix with the honey solution. Tip the reaction vessel 4-5 times to insure a thorough mixing, but do not remove from the water bath during this step. Note the time of mixing.

Periodically remove 0.5 ml. of the digestion mixture and quickly add it to 5 ml. of the iodine solution. Note each time of sampling. It is assumed that hydrolysis stops on contact with the iodine. A cotton-stoppered pipette will allow rapid removal and delivery of the 0.5 ml. sample.

Add amount of distilled water found to be required for standardizing the reaction mixture, and immediately determine relative color intensity of sample with the colorimeter. Use distilled water as a blank to zero the instrument.

The color contributed by the iodine and the honey used can be determined readily but is negligible because of the dilute solution used, e.g., 1 to 2 Klett units. It is permissible to apply this correction factor to all the readings or to omit the correction altogether.

Expression of activity. Convert colorimeter readings to transmittance values if the instrument is not scaled in these units. Then plot the transmittance values against time as the abscissa. This results in a sigmoid curve which is essentially straight-line in the range 35 to 70% transmittance. From the plot, determine time required for the reaction mixture to yield a 50% transmittance value. Only 3 to 4 points within the range 35 to 70% transmittance need to be plotted to accurately fix this time.

The unit of diastase activity established for the method is that amount of enzyme which will convert 0.01 g. of starch to the prescribed endpoint in 1 hour under the test conditions. Results are expressed in units per gram of honey.

Since in the reaction mixture 0.1 g. of starch is converted by 2.0 g. of honey, the units per gram of honey equals 300 divided by the number of minutes (t) required to reach the 50% transmittance value, or units per gram $= \frac{60}{t} \times \frac{0.10}{0.01} \times \frac{1.0}{2.0} = \frac{300}{t}$

(b) *Determination of HMF*: A qualitative test for the presence of HMF was made in a manner similar to that used by Fiehe *(5)*. The extraction of HMF with ether was standardized in a manner described by Dr. H. Duisberg, who personally illustrated the test to the authors.

The first approach used to determine the HMF concentration in honey was by estimation from the U. V. spectral absorption curves of dilute solutions of honey. Although

reasonably reliable results could be obtained it seemed desirable to separate the HMF from other substances in the honey that absorb in the region of 270 mμ, or at least to concentrate the HMF in respect to such substances, by ether extraction. A uniform procedure for extraction was employed that permitted acceptable duplication of the results. The ether extract was allowed to evaporate to dryness at room temperature and the residue was mixed with water. A Beckman DR Monochromator was used to establish the U. V. absorption curves.

This spectrophotometric procedure was especially useful for studying changes that occur in honey during storage. Accurate estimates of the HMF content could not be made, however, since some of the interfering substances also proved to be ether extractable.

The work of Winkler *(13)* appeared after the major portion of the present work was completed. His procedure for the spectrophotometric determination of HMF in honey was only tested in comparison with the method proposed below and not used routinely in this study.

A modification of the Fiehe test was devised that uses a photoelectric colorimeter to measure objectively the color intensity of the Fiehe reaction. It was shown that the intensity of the color is proportional to the HMF concentrations to at least 175μg./ml.

Reagents and Apparatus

(a) *Resorcinol solution.* Dissolve 0.50 g. of resublimed resorcinol in 50 ml. HCl (sp. gr. 1.19).

(b) *Alcohol.* 95% ethanol.

(c) *Ether.* $(C_2H_5)_2O$, anhydrous; Reagent grade.

(d) *Colorimeter.* Klett-Summerson photoelectric colorimeter, Model No. 900-3, fitted with No. 54 green filter, or similar instrument.

Preparation of Standard Curve

Standard aqueous solutions are prepared with hydroxmethylfurfural (HMF) obtained according to the method of Haworth and Jones [4]. The concentration of HMF in the standard solutions should be checked spectrophotometrically. The molecular extinction coefficient of pure HMF at 285 mμ is 16,500 [5]. The standard solutions should be in the range of 0 to 50 mg/liter, since higher concentrations are not usually found in honeys unless they have been severely heated.

To 3 ml. of each standard solution add 3 ml. of ethanol and mix. Then add 3 ml. of the resorcinol solution and mix thoroughly by shaking. Allow the mixture to stand at room temperature for exactly 30 minutes after the addition of resorcinol; then determine the color intensity against distilled water with the colorimeter fitted with a No. 54 green filter. (The color complex shows maximum absorption at 490 mμ.) Plot the Klett readings against the concentrations of the standard solutions (μg HMF/ml.). Beer's Law is obeyed to a concentration of at least 175 μg. HMF/ml.

Determination

(a) *Extraction of sample.* Weigh accurately 5.00 g. of honey into a mortar (approx. 7 cm. diam.; Coors, U.S.A., No. 520-2). Using a pestle, grind the honey for 2 minutes with 10 ml. of ether; decant the ether extract into a small evaporating dish. Repeat the extraction twice in the very same manner and collect the extracts in the same dish. Allow the combined extracts to evaporate to dryness at room temperature and then promptly add 5 ml. of distilled water. With honeys containing sufficient HMF to give a weakly positive Fiehe reaction about 80-85% of the HMF is extracted by this procedure. Mix the residue of the extract with the distilled water using a stirring rod and filter the resulting solution through Whatman No. 1 paper (9.0 cm.) to remove the insoluble substances. Collect all the filtrate possible, since there is just enough for one HMF determination.

(b) *Measurement of HMF in extract.* Using 3 ml. of the aqueous solution of the extract follow in detail the procedure described for preparation of the standard curve.

[4] Haworth, W. N. and Jones, J.K.N. *J. Chem. Soc.*, 667 (1944).

[5] Wolfrom, M. L., Schuetz, R. D., and Cavalieri, L. F. *J. Am. Chem. Soc.* **70,** 514 (1948).

Tube No. (Plate No.)	Volume of honey solution ml.	Volume of sterile water ml.	Ca. percentage of honey in final agar medium (w/w)
1	10	3	21.3
2	8	5	17.3
3	6	7	13.1
4	4	9	8.9
5	2	11	4.5

Estimate the HMF concentration in the extract from the standard curve. Since the residue from 5 g. of honey is dissolved in 5 ml. of water, the μg of HMF per gram of honey is read directly from the curve.

(c) *Determination of "inhibine value"*: The inhibiting property of honey toward bacterial growth was estimated with a modification of the method proposed by Dold and Witzenhausen [1]. In the modified method a series of 5 plates of nutrient agar is prepared with increasing amounts of honey ranging from ca. 4.5 to 21.3%. The higher concentrations, 25-26%, used by Dold and Witzenhausen were not used because the inhibition by "inhibine" could not be distinguished easily from that of sugar at high concentration. A system of relating the tube and plate number to the inhibine value was also devised which simplifies record keeping and recording of results.

Procedure

Double strength nutrient agar medium (Difco) is prepared and dissolved in water by heating. Cool to 60-65° C. and pipette 15 ml. portions into screw-cap test tubes. Autoclave the tubes for 15 minutes at 15 pounds pressure.

Carefully weigh out 25 g. of honey into a sterile wide-mouth jar of at least 125 ml. capacity. Add 25 ml. of sterile water and dissolve the honey rapidly at room temperature with shaking. Five dilutions of this sample are prepared by pipetting the volumes of honey solution and sterile water, shown in the table below, into sterile test tubes.

Liquefy the tubes of agar in a water bath and allow them to cool to 50° C. Warm the tubes of diluted honey solutions for 1-2 minutes in a water bath controlled never to exceed 40° C. Carefully add the entire contents of a tube of honey solution to a tube containing the sterile agar. Mix the agar and honey solution thoroughly by pouring back and forth between the tubes 7-8 times. Then pour two petri plates immediately (12 ml./plate).

Inoculate each plate with 0.1 ml. of a sterile-water suspension of *S. aureus* prepared from a 20-30 hour culture grown on a nutrient agar slant at room temperature. (Strain No. 170 of the Department of Food Technology collection was used). Place the inoculum at the center of the plate and spread with a glass rake. The growth is observed and recorded after incubation for 20-24 hours at 20° C.

The inhibine value is determined from the plate of lowest honey concentration that shows inhibition of growth of the test organism. The inhibine value is arbitrarily assigned the number of the plate showing such inhibition, since the plates are numbered inversely to the concentration of honey present. If instead of complete inhibition a plate shows sparse growth or only a trace of growth ($\pm$), the inhibine value is estimated to be some fraction less than that of the plate showing such poor growth.

RESULTS AND DISCUSSION

Five samples of comb honey from several floral sources and 7 samples of commercially extracted honeys from similar floral sources were examined for diastase activity with both of the methods described above. As shown in Table 1, the activities obtained by the two methods agreed remarkably well, especially in view of the notable differences that exist between the methods. The photoelectric method is obviously preferable on the basis of objectivity, reproducibility, and precision; it was used for all following experiments.

[1] Information concerning this method was provided by Dr. H. Duisberg.

TABLE 1
Comparison of new photometric method with a modified Gothe method

No.	Type of honey	Diastase activity	
		Photometric method	Gothe method
1	Alfalfa, extr.	12.0	14
2	Cotton, extr.	24.6	24
3	Mesquite, extr.	11.3	11
4	Buckwheat, extr.	27.5	—
5	Orange, extr.	5.8	—
6	Sage, extr.	14.8	>14
7	Alfalfa, comb	9.9	> 8.3
8	Orange, comb	4.0	> 2.5
9	Buckwheat, comb	20.0	>>18
10	Alfalfa, comb	22.4	23
11	Alfalfa, comb	23.1	18
12	Mixed, extr.	5.0	5

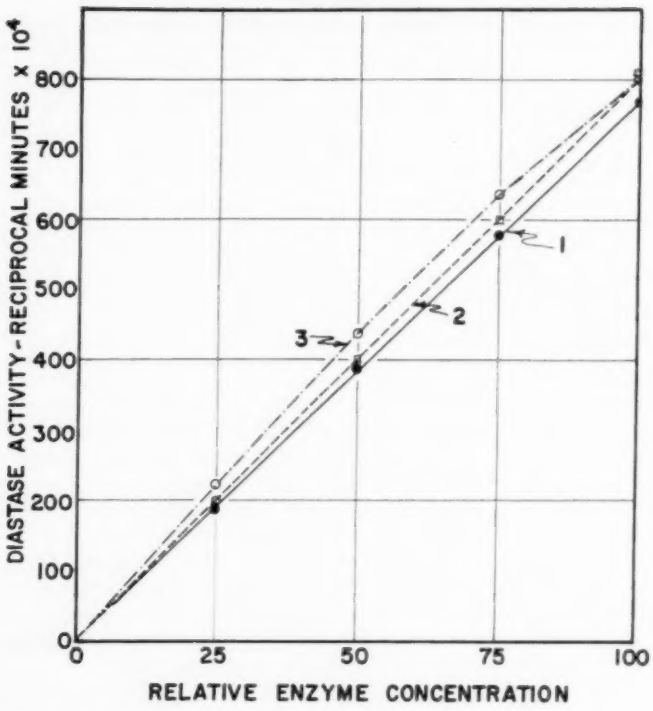
This method also involves less glassware and often less time, since a single dilution of honey is used per assay rather than a series of several dilutions. A marked advantage of this method is that a precise measurement of the diastase activity is obtained by a single assay, whereas methods employing a series of dilutions of honey often require a preliminary assay to determine the proper range of dilutions for an accurate estimate.

As can be noted in Figure 1, the concentration of sugars in the assay mixture seems to influence the diastase activity somewhat; therefore, theoretically at least, it is preferable to conduct such assays under a condition of nearly constant sugar concentration, so that this effect can be disregarded. This condition is not obtained in methods like that of Gothe.

The same samples were examined for HMF using the Fiehe Test and the U.V. absorption of aqueous solutions of the honeys and of aqueous solutions of the residue of ether extracts of the honeys. Absorption spectra of aqueous solutions of honeys were of little value for measuring small amounts of HMF because of the presence of variable amounts of interfering substances that mask the spectrum of low concentrations of HMF. On the other hand, the ether extraction procedure yielded absorption spectra that corre-

TABLE 2
Comparison of the Fiehe reaction and U.V. absorbance of the ether extract

No.	Fiehe reaction	Maximum absorbance		HMF (approx.) μ/gm.
		272 mμ	284 mμ	
1	+		0.360	13.8
2	− −	0.540		
3	+ +		0.570	21.8
4	−	1.250		
5	+ −		0.246	9.4
6	+ −		0.140	5.3
7	−		0.075	2.9
8	− −		0.030	1.1
9	− −	0.440		
10	− −	0.480		
11	− −	0.205		
12	+ + +		0.730	27.9



Legend:
1 = Diastase from honey prepared by ethanol precipitation.
2 = Honey solutions diluted to lower concentrations with inactive honey.
3 = Honey solutions diluted to lower concentrations with water after adding NaCl to maintain a uniform concentration of NaCl.

Figure 1. Check on the validity of the modified method as an assay of diastase activity of honey.

lated fairly well with the Fiehe reaction of these samples; c.f., Table 2. However, in some cases the extraction procedure still resulted in solutions containing sufficient amounts of interfering substances to mask the presence of low concentrations of HMF.

Samples giving positive Fiehe reactions were always among those honeys giving spectra with maximum absorption at 282-4 mμ and minimum absorption at or near 245 mμ, which is similar to the spectrum of HMF. As shown in Table 2, optical densities of the maxima at 284 mμ of such samples showed good correlation with the intensity of the Fiehe reaction. The approximate concentrations of HMF in the samples were calculated using the molecular extinction coefficient for solutions of pure HMF, E = 16,500. The estimation of HMF in sample number 1 indicates that a honey containing approximately 13.8 micrograms of HMF per gram will give a positive Fiehe reaction.

Samples were stored in closed jars in a constant temperature room at 20° C. for 13 to 15 months and then examined again for diastase and HMF.

TABLE 3

Comparison of diastase and HMF before and after storage for 13 to 15 months at 20° C.

	Diastase (units/gram)		Fiehe reaction		Change in HMF Estimated from the change in spectrum of ether extract (micrograms/gram)
	Before	After	Before	After	
1	12.0	11.5	+	++	32.9
4	27.5	26.1	−	−+	——
5	5.8	5.2	+−	+	6.1
6	14.8	14.0	+−	+	10.2
7	9.9	7.4	−	+	7.0
8	4.0	——	−−	−+	——
9	20.0	16.6	−−	−	——
10	22.4	21.6	−	+	——

The diastase activities and Fiehe reactions of several samples before and after storage are compared in Table 3. After storage at 20° C. for 15 months, the diastase activity had decreased slightly, but not significantly in most cases. On the other hand, the HMF had increased sufficiently during storage to cause significant changes in the Fiehe reaction as well as in the U.V. absorption of the ether extractable material; c.f., Figure 2. An estimation of the increase in concentration of HMF was made from the change in absorption spectra of those samples showing maximum absorption near 284 mμ before and after storage. The results are included in Table 3; it should be noted that the greatest increase in HMF was found in sample number 1, which was quite positive in HMF before storage.

In addition to the natural variation of diastatic activity in fresh honeys, Kiermeier and Koberlein *(7)* reported a variation in sensitivity of diastase to heat among honeys and concluded that the latter variation is due to the natural differences in pH among honeys. The present work is in agreement with their findings.

Data obtained for the heat inactivation of diastase in various honeys illustrates the precision of the method developed to measure the diastatic activity of honey; c.f., Figures 3 and 4. As was to be expected first order kinetics were observed for the inactivation in every case. The alfalfa honey (M in Figure 4) was also heated at several other temperatures to study the effect of temperature on the rate of inactivation of diastase; c.f., Figure 5. From the slopes obtained in Figure 5 an Arrhenius-type plot was made to relate the rate of inactivation to the temperature; c.f., Figure 6. The actual rate constants (k) for the inactivation of diastase are not obtained from the slopes in Figures 3, 4 and 5, since enzyme activity is used instead of enzyme concentration. Instead, the slope of each line in these plots when multiplied by 2.3 gives an "apparent rate constant" for inactivation that is equal to the actual rate constant, k, multiplied by a constant quantity, K, that may be defined as a proportionality factor relating enzyme activity to enzyme concentration. Hence, a plot of the "apparent rate constants", kK, against the reciprocal of the absolute temperatures results in an Arrhenius-type plot that enables one to estimate the rate of inactivation at intermediate temperatures. Such data collected for many honeys could prove of tremendous value in studying the factors affecting the rate of loss of diastase in different honeys.

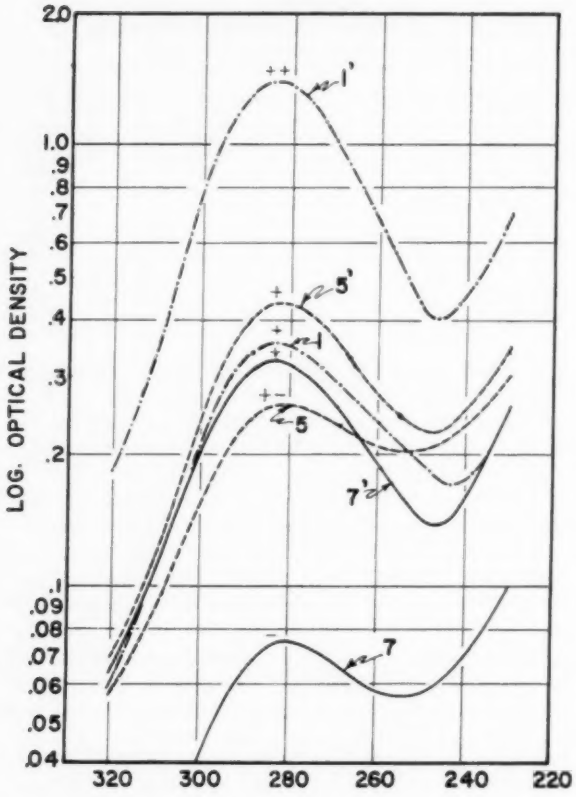


Figure 2. Changes in the absorption spectra of other extractable material during storage at 20° C. for 15 months.

The following analysis is an example of the application of the information obtained from the Arrhenius-type plot. The "apparent rate constants", kK, for the inactivation of diastase at 71° C. and 65° C. were calculated from the curves in Figures 3 and 4 were plotted against pH to determine the effect of pH on inactivation; c.f., Figure 7. The "apparent rate constant" for the inactivation of diastase in honey M at 65° C. was calculated from the Arrhenius-type plot in Figure 6, because this honey was not heated at 65° C. The calculated kK, as shown by the double circle in Figure 7, falls within the range of values obtained for honeys of similar pH. Figure 7 further illustrates that the increase in rate of inactivation with increase in temperature from 65° C. to 71° C. is greater for honeys of low pH than for those of high pH. Thus, the data seem to substantiate the hypothesis that sensitivity of diastase to heat varies among honeys because of differences in their pH.

The photoelectric colorimetric measurement of HMF was devised to overcome the subjectiveness of the old Fiehe test. Although the undesirable

extraction with ether is still involved in this method, the measurement of the intensity of the Fiehe reaction with the colorimeter allows a quantitative estimation of the HMF present in the extract.

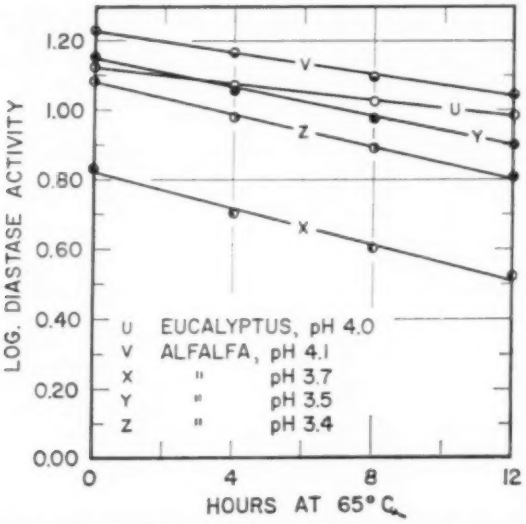


Figure 3. Inactivation of diastase in honeys stored at 65° C.

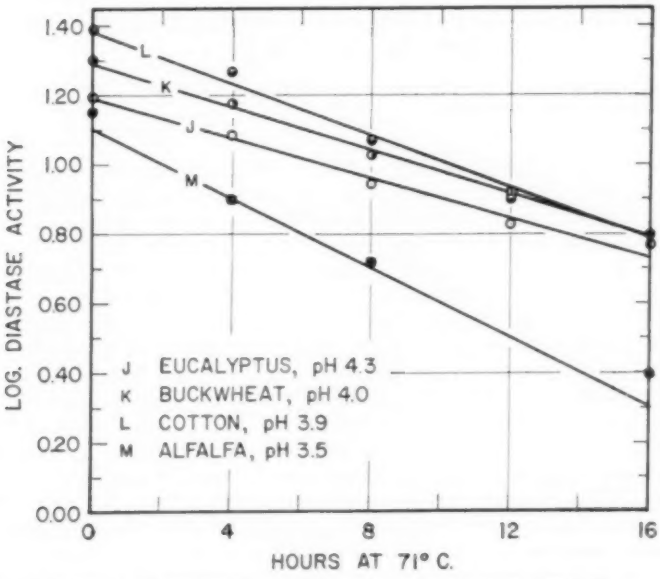


Figure 4. Inactivation of diastase in honey stored at 71° C.

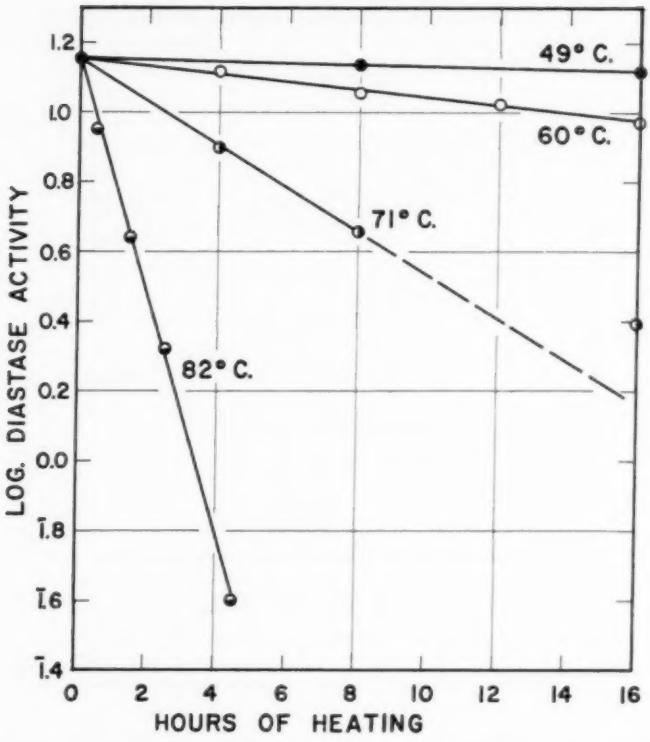


Figure 5. The effect of temperature on the loss of diastase activity in an alfalfa honey (pH 3.5; 14.5% moisture).

An example of the application of both the photoelectric colorimetric method for diastase and that for HMF is shown in Figure 8, which illustrates clearly the effect of heat on diastase and HMF in a given alfalfa honey. The results show that this honey became very low in diastase activity before it contained sufficient HMF to give a "positive Fiehe reaction" with the qualitative test, which detects HMF only in concentrations above 7-9 μg/g. as determined with the "quantitative test". Figure 8 also shows that the rate of forming HMF increases as the HMF content increases. It should be noted that the change in rate is even greater than that shown here, the efficiency of the extraction procedure decreases as the HMF content increases.

Moisture content is one of the factors that influences the rate of HMF formation. To study the effect of moisture content, samples of a single white alfalfa honey (approximately 12.5% moisture) were diluted with increasing amounts of distilled water to moisture contents ranging up to 17.2%. These samples were then heated at 55° C. for 1, 2, and 6 days. The moisture contents were determined with an Abbe refractometer and a Chataway table before and after the heat treatments. As usual 5.00 g. of the samples were

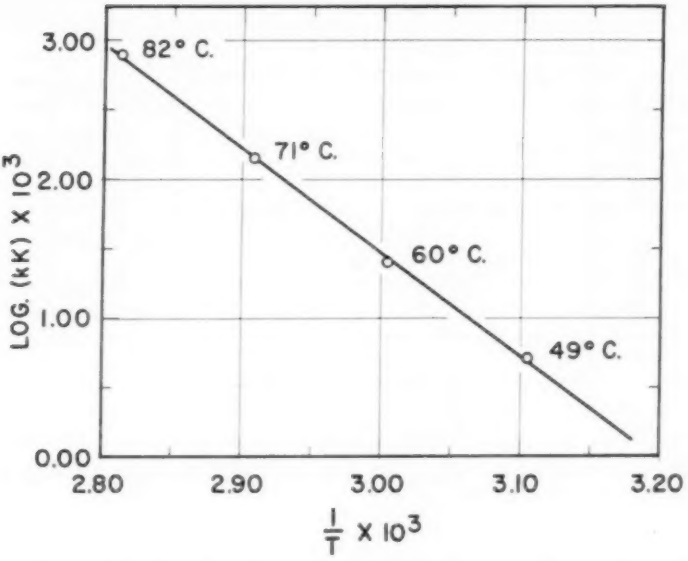


Figure 6. An Arrhenius-type plot relating rate of inactivation of diastase with temperature for the data shown in Figure 5.

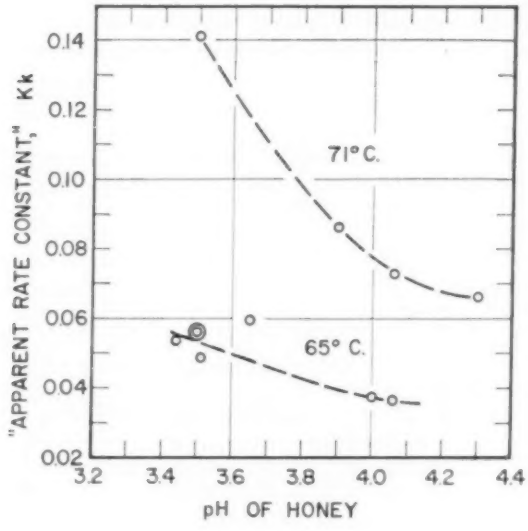


Figure 7. The relationship of the pH of honey to the rate of inactivation of diastase in honeys heated at 65° and 71° C.

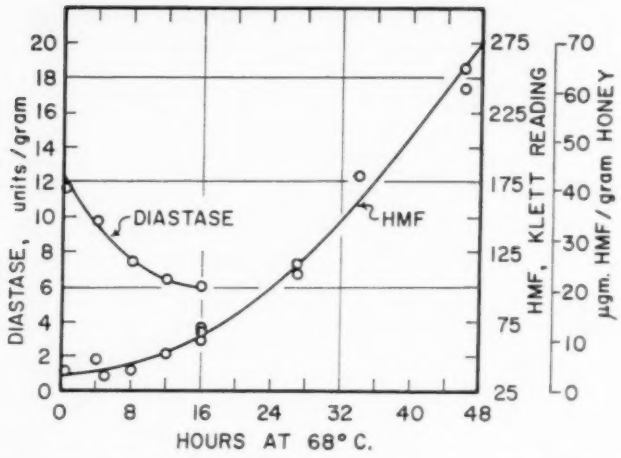


Figure 8. The effect of heat on diastase and HMF concentrations in an alfalfa honey (pH 3.7; 16% moisture).

extracted with ether for the analysis. The concentration of HMF was calculated from the assay for the weight of the original honey represented in 5.00 g. of the adjusted samples. The amount of HMF formed in 6 days at 55° C. increased notably with increase in moisture; c.f., Figure 9. The difference was much less marked in the less severely heated samples.

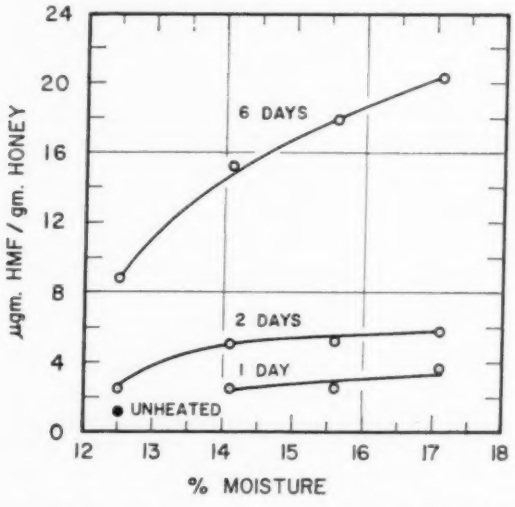


Figure 9. The effect of moisture content on the rate of HMF formation in an alfalfa honey heated at 55° C.

In another experiment a second sample of white alfalfa honey was used to prepare a series ranging in moisture contents from 11.4% to 20.7%. These samples were heated for 6 days at 55° C., stored for 3 weeks at room temperature, and then assayed for HMF. For this experiment the amount of each sample used for the assay was proportional to the moisture content and was equivalent to 5.00 g. of the original honey. Besides the usual 3 extractions of each sample with 10 ml. aliquots of ether, four more extractions were made with 5 ml. aliquots of ether to obtain more complete and uniform extraction; thus, the total time of extraction was 14 minutes. For better and easier extraction the samples of low moisture content were adjusted to about 17-18% moisture by addition of proper amounts of distilled water to the samples in the mortar. In addition to the quantitative Fiehe test devised, the p-toluidine method of Winkler *(13)* and the change in the spectra of aqueous solutions of the honey were used to estimate the concentration of HMF in the heated samples. For the spectral measurements, aqueous solutions of honey were used in a concentration equivalent to one gram of the original honey per 50 ml. of solution. As noted before, interfering substances make it impossible to determine the amount of HMF from the absorbance of aqueous solutions of honey. For this reason three possible methods of analyzing the spectra were employed. First, the increase in absorbance of 284 mμ during the heat treatment was used to calculate the increase in HMF; for this estimate the unheated honey was assumed to contain no HMF. Second, the change in the difference between the absorbance at 284 mμ (maximum) and that at 245 mμ (minimum) was calculated for all samples. Then an extinction coefficient was estimated for the value ($E_{284} - E_{245}$) with a standard solution of HMF and used to estimate the amount of HMF present in the various samples[5]. Third, the spectral data were analyzed with a formula similar to that proposed by Winkler for analyzing clarified solutions of honey.

Results of all methods are compared in Figure 10. All methods demonstrated the importance of moisture on the rate of HMF formation. In general the difference in the concentration of HMF between low moisture and high moisture samples was roughly the same by all methods, but the concentration of HMF in a sample varied greatly with the method of analysis.

Results with the quantitative Fiehe reaction were consistently and proportionately lower than those obtained with the p-toluidine method of Winkler. The very low reading obtained with the quantitative Fiehe test for the sample of 20.6% moisture is easily explained by the difficulty experienced in grinding this sample in the mortar with ether; the grinding action was noticeably poor because of the high moisture. The difference noted between the results of these two chemical methods seems to be greater than that to be expected from the incomplete extraction of HMF in the quantitative Fiehe test. The notably lower concentrations obtained with the quantitative Fiehe reaction might be due to an increase in specificity of measurement brought about by the ether extraction involved in this method.

Rough estimates of HMF concentration from the increases in absorbance at 284 mμ during the heat treatment were higher than those obtained with the chemical methods, showing that interfering substances also are produced during heat treatments of honey.

[5] The extinction coefficient for $(E_{284} - E_{245})^{1\%/1\,cm} = 122$.

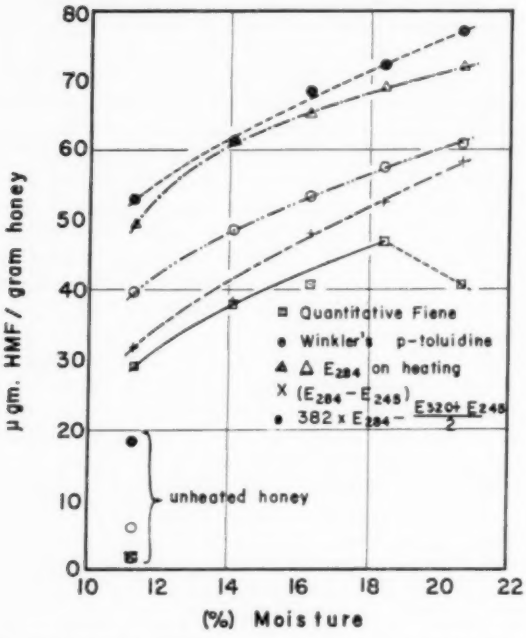


Figure 10. The effect of moisture on the formation of HMF in a honey heated at 55° C. for 6 days.

It is interesting to note that the result obtained by estimating the HMF from the value ($E_{284} - E_{245}$) fell between those obtained by the two chemical methods, and therefore seems to be a fairly good estimate of HMF concentration in this honey.

Analysis of the spectra with a formula similar to that of Winkler gave results that paralleled those of the previous spectral analyses but which were consistently higher than the previous methods. Winkler also reported that high results are obtained if unclarified solutions of honey are analyzed with such a formula.

The above data indicate a lower specificity of the p-toluidine-barbituric acid method for HMF than that found with the quantitative Fiehe test described in this paper. However, the simplicity of the p-toluidine-barbituric acid method might well overshadow the lower specificity indicated for it.

An experiment was made to compare the rates of formation of HMF in 3 alfalfa honeys that had similar moisture contents (16.6, 16.6, 16.1), similar pH values (3.7, 3.6, 3.7), and no appreciable amount of HMF. Samples of the honeys were stored in incubator rooms at 35° C., 45° C., and 55° C. after bringing the samples to the desired temperatures in water baths. As shown in Figure 11, the rates of forming HMF varied measurably among these honeys, although the differences were not great until the heat treatments were prolonged and objectionable amounts of HMF had been produced. Since

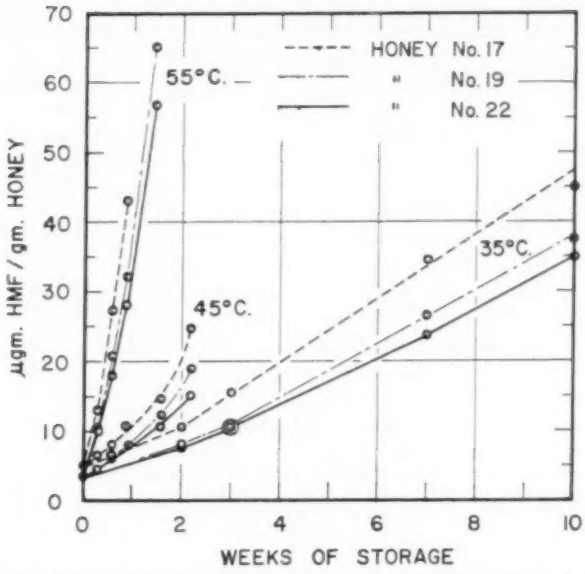


Figure 11. Comparison of the rate of formation of HMF in three alfalfa honeys during storage at elevated temperatures.

such temperatures are within the range of climatic temperatures commonly reached in some parts of California, it appears quite possible that detectable amounts of HMF could be formed by comparatively short exposures to solar heat.

The data presented indicate that the rate of forming HMF and the rate of losing diastase activity may vary greatly among different honeys even when they are treated in similar fashion in respect to heat and storage. In view of such variation it seems likely that the establishment of minimum diastase contents or maximum HMF contents could discriminate against certain honeys that are heated to no greater extent than the others found acceptable under such standards. The relationship of the nutritious values (e.g., inhibine) of honeys to such standards remains to be studied. With the latter question in mind a few preliminary examinations were made to compare the "inhibine values" of honeys that did and did not meet the present rules for judging honey employed by the Instituts fur Honigforschung in Bremen, Germany. The diastase activities and the Fiehe reaction and/or HMF contents of "acceptable" and "unacceptable" honeys are compared with their inhibine values in Tables 4 and 5.

Honeys in Table 4 have too much HMF for their comparatively low diastase activities to meet present German standards. Nevertheless, honey No. 57 which had a considerable amount of HMF gave the maximum inhibine value with the present procedure. Furthermore, this inhibition was shown to be due to a heat labile factor, since the inhibition was lost completely when the 50-50 solution of honey in water was heated in a boiling water bath for 15-

TABLE 4

The "inhibine values" of several honeys that fail to meet the German regulations for diastase and HMF.

No.	Honey	Fiehe Reaction	HMF quantitative Fiehe method (μgm./gm.)	Diastase (units/gm.)	Inhibine	
					Assay	After heating honey sol'n.
9	Alfalfa, 1955	+ −	9.0	6.7	4.0	
10	Alfalfa, 1955	+	13.0	6.9	4.0	1.0
55	Alfalfa, 1955		15.0	4.5	4.0	
57	Alfalfa, 1955		18.0	6.7	5.0	0.0
58	No. 57-liquid		33.0	trace only	0.0	
Q	Alfalfa, 1954	+ + +	——	7.3	3.0	
5x	Orange, 1954	+	——	5.2	0.0	
7x	Alfalfa, 1954	+	——	7.4	1.0	

TABLE 5

The "inhibine values" of several honeys that apparently meet the German regulations for diastase and HMF.

No.	Honey	Fiehe Reaction	HMF quantitative Fiehe method (μgm./gm.)	Diastase (units/gm.)	Inhibine	
					Assay	After heating honey sol'n.
20	Alfalfa, 1955	−		17.1	4.0	1.0
M	Alfalfa, 1954	−	4.5	12.5	4.0	
M°	No. M, heated[1]		7.0	9.5	0.5	
4x	Buckwheat, 1954	− +		26.1	0.5	
10x	Alfalfa, 1954	+		21.6	2.5	

[1] Heated over a 3 hour period to 71° C.

20 minutes. It seems noteworthy that the buckwheat honey No. 4x in Table 5 showed a very low inhibine value in spite of its very high diastase activity and low HMF content.

The results with honey M in Table 4 and with No. 57 in Table 6 indicate that the inhibine is more sensitive to high temperatures than diastase. If the inhibine is more sensitive to heat than diastase, the honeys in Table 4 that have high inhibine should not be judged unacceptable because of alleged overheating. The search for better methods of judging the acceptability of a honey is now in progress in Germany. It is our hope that this paper will be of some aid in this pursuit.

TABLE 6

A comparison of the effect of heat (62.8°) on inhibine and diastase in honey number 57. Stored for 18 days before analyzing

Treatment	Inhibine value	Diastase units/gm.
Unheated	5.0	6.7
0 Hour		
15 Min. Preheating	4.5	
1 Hour	3.0	
4½ Hours	2.3	5.4

SUMMARY

Objective methods of measuring the diatase (α-amylase) activity and HMF content of honey with the aid of photoelectric instruments were developed and employed to study the occurrence of these substances in unheated honeys. The well-known variation in diastase activity among different honeys was demonstrated. Evidence was given to support Kiermeier and Koberlein's theory that the variation in sensitivity of diastase of different honeys to heat is due to the variation in pH among honeys. The rate of formation of HMF was shown to vary among honeys under similar treatments of heat and storage and to be greatly influenced by the moisture content of the honey and by the concentration of HMF initially present. Various methods of estimating HMF concentration in honey were compared. The heat labile bactericidal substance in honey ("inhibine") was shown to be more heat labile than diastase. No relationship between diastase activity, HMF content, and inhibine value could be found in several honeys that could and could not meet the present German requirement for diastase and HMF.

Acknowledgment

The authors are indebted to Dr. Herworth Duisberg, Leiter des Institut fur Honigforschung, for his most valuable description of the methods used in Germany, for his help in orienting the authors in respect to the German viewpoint on the subject, and for his actual assistance in certain experiments.

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A STUDY OF THE BIOCHEMISTRY OF IRRADIATED POTATOES STORED UNDER COMMERCIAL CONDITIONS[a]

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One of the early successful applications of irradiation in the preservation of foodstuffs was the inhibition of sprout formation in potatoes as outlined by Sparrow *et al. (18, 20, 21)*. In work reported by Sparrow and Christensen *(19)* sprouting was greatly reduced at dosages of 5,000 rep. and completely inhibited at 20,000 rep. or higher.

Sawyer *(15, 16)* found that dosages of around 10,000 rep. or higher completely inhibited sprouting regardless of the storage temperature. Brownell *(3)* reported dosages of from 10,000 to 15,000 rep. as minimums necessary for complete sprout inhibition.

In more recent work Burton and Hannan *(5)* reported sprout suppression at much lower dosages of 3,500 to 8,500 rep.

Along with the beneficial effect of sprout inhibition has come an undesirable effect of rotting of the tubers after irradiation at the higher levels. Brownell *(3)* found that following dosages of 19,000 to 200,000 rep. periderm formation was inhibited and suberization was delayed. Therefore, under certain conditions rot could occur as a result of infections in cuts and bruises which occur after irradiation. If bruising occurred and the samples were irradiated at the higher dosages before the tubers had a chance to form periderm, these bruises would then offer likely entrances for rot-causing organisms.

The effect of irradiation upon ascorbic acid has been investigated by several workers. Proctor and Goldblith *(13)* in studying the effect of X-rays produced at 50 KV on pure solutions found ascorbic acid to be more radiosensitive than either niacin or riboflavin. Anderson *(1)* found that half of the ascorbic acid content was destroyed when solutions of approximately 4 mcg. per ml. were exposed to 5,500 rep. of X-rays produced at 185 KV.

In complex systems ascorbic acid losses are somewhat less than in pure solutions. Proctor and O'Meara *(14)* found considerable loss of ascorbic acid in orange juice irradiated by high voltage cathode rays. Kung and Gaden *(7)* found that ascorbic acid was destroyed to a considerable degree when evaporated milk, cream, cheese, butter and margarine were exposed to ionizing radiation dosages of 8×10^4 rep. to 2×10^6 rep. for periods of 1 to 12 hours. Both Brownell *(4)* and Sawyer *(15)* have shown losses of reduced ascorbic acid in irradiated potatoes. Sereno *(17)* indicated an initial loss of ascorbic acid immediately after irradiation, followed by an arresting of this

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loss, and later by a gradual recovery of the vitamin. This recovery was accelerated by high storage temperatures, i.e. the higher the storage temperature the sooner the recovery. Similar instances of this ascorbic acid recovery have been reported by Cooke *(6)* in his work with irradiated snapdragon plants and soya bean plants.

This study was undertaken to determine the changes occurring in Maine grown Katahdin and Russet Burbank and Idaho grown Russet Burbank potatoes when irradiated at two levels and stored under commercial conditions for 13 months. Each month analyses were run to determine the moisture content, specific gravity, total sugars, reducing sugars, ascorbic acid and starch. The respiration was determined bimonthly for the first 8 months only. A record of the temperatures during the storage period was also kept.

MATERIALS AND METHODS

Sample treatment. The two Maine varieties were shipped by rail to the irradiation facility at Idaho Falls, Idaho. Approximately 5,000 pounds received no treatment, 7,500 pounds received irradiation dosages of 14,000 to 20,500 rads and approximately 10,000 pounds received dosages of 28,000 to 41,000 rads. The Idaho Russet Burbanks were treated in a like manner. Each lot, however, was comprised of approximately 1,000 to 2,000 pounds more potatoes than the corresponding Maine lots. After irradiation the samples were shipped to Fort Fairfield, Maine, and stored in a commercial potato storage house.

Specific gravity. The Parow Potato Balance *(12)* was employed using a 5 kg. sample whenever possible and smaller samples when necessary.

Reducing sugar. The AOAC Berlin Institute method *(2)* was used with the following modifications suggested by Ogg *(11)*. Eight to 10 unpeeled potatoes were cut from stem to bud end and 200 g. of opposite radial 1/16 sections were comminuted in a Waring blender with an equal weight of water for 5 minutes. One hundred grams of the slurry was transferred to a beaker, and 1 to 2 g. calcium carbonate and 250 ml. of 95% ethyl alcohol were added. The mixture was extracted on a steam bath for 1 hour after which the solution was decanted and filtered. The residue was similarly extracted two additional times using 76% alcohol (200 ml. of 95% alcohol plus 50 ml. water) and filtered as before.

Filtrates were evaporated to approximately 450 ml., transferred to 500 ml. volumetric flasks and made up to volume with water. The procedure outlined by the Berlin Institute method was used to determine the reducing sugars.

Total sugars. A 100 ml. aliquot of the alcoholic extract prepared for reducing sugars was hydrolyzed 24 hours at 20°-25° C. with 20 ml. of 7.8 N hydrochloric acid. It was made slightly basic with 5 N potassium hydroxide using phenolphthalein as an indicator. The hydrolyzed sample was made up to volume in a 200 ml. volumetric flask and the same procedure used as for the determination of the reducing sugars *(2)*.

Moisture. Five grams of the same slurry that was used in the extraction of the reducing sugars was used for this determination. The sample was dried for 24 hours at 75° C. and at atmospheric pressure.

Ascorbic acid. A method adapted from Loeffler and Ponting *(9)* was employed. Unpeeled opposite radial sectors from stem to bud end of 5 or 6 potatoes were used. Fifty grams of sample was comminuted in a Waring blender with 350 ml. of 1% metaphosphoric acid for 5 minutes. The extract was filtered through Whatman No. 42 filter paper and the ascorbic acid was determined on the filtrates using a Klett-Summerson photoelectric colorimeter with a No. 54 green filter.

Starch. Starch determinations were made on the residue recovered from the alcoholic extraction prepared for the determinations of the sugars. The AOAC method for starch was used *(2)*.

Respiration. Five-pound lots of potatoes were placed in sealed bell jars. Air free of moisture and carbon dioxide was drawn through the jars by means of a vacuum pump. The moisture and carbon dioxide given off by the potatoes during respiration was collected in U-tubes containing Drierite and Ascarite respectively. Weight gains in the U-tubes were accepted as the water and carbon dioxide given off by the tubers during respiration.

RESULTS AND DISCUSSION

For the purpose of this study the Russet Burbank potatoes from Idaho and those from Maine were considered as two different varieties. Therefore, later statements will refer to "3 varieties." All values indicated in this report are on a fresh potato basis and not on a moisture-free basis.

All of the controls sprouted, but sprouting of the irradiated samples was completely inhibited with the following exceptions. At the end of 6 months' storage sprouting occurred in approximately 50 pounds of low dosage irradiated Katahdin samples. At the end of 7 months some sprouting was evident in the irradiated low dosage Idaho Russet Burbank lot. Approximately 5 to 10 pounds were affected in this instance. As previously noted Burton and Hannan *(5)* were able to suppress sprouting using one-fourth to one-half the low dosage employed in this study. Their dosage rates should be examined with the fact in mind that Burton did not use any of the common American varieties which possibly may require a higher dosage for sprout suppression than the varieties they used.

The most significant result of this study was the much greater incidence of rot in the irradiated samples than in the controls, as can be seen in Table 2. Except for the very end of the storage period the percentages of rot were much higher for the irradiated than for the controls. Lowering of the percentages rot for both low dosage irradiated Russets toward the latter phase of storage and the corresponding increase in rot percent for the controls during the same period would seem to indicate that the irradiated tubers that did not rot early in storage were less susceptible to rot towards the end of the storage period than were the controls. The average cull figures indicate that Katahdins were slightly more susceptible to irradiation damage than either Russet variety, the Idaho Russet being least susceptible. Averaging the overall rot percents for the 3 control lots and then comparing that average against the low dosage lot average and against the high dosage lot average it appears that irradiation at the low dosage resulted in 29.0% more rot than the controls and the high dosage irradiation resulted in 33.0% more rot than the controls. The inhibition of periderm formation as reported by Waggoner *(22)* added to the delay of suberization as reported by Brownell *(3)* very probably is responsible for the increase in rot in the irradiated samples in this study. The added handling necessary at the irradiation site itself probably played a part in this increased rot.

A check to determine if the added handling due to shipping the Maine grown varieties to Idaho and back to Maine had any effect on the rotting is limited to an examination of the first half of the storage period only. This is so because potatoes in Maine are usually stored about 7 months; therefore normal rot figures are available for this length of time only. Making this comparison it appears that both Maine control lots exhibited about twice the amount of rot normally encountered in storage in Maine. It then follows that part of the rot of the irradiated samples was due to the added handling because of the shipping.

Figures 1, 2, and 3 present the total sugars, reducing sugars and starch contents throughout the study. It appears that the temperature of the storage had a much greater effect on these values than did the irradiation treatment. During the warmer months of storage there was a reduction in the

TABLE 1

Temperature of Storage (°F.)

	Storage Time in Months												
	Jan 57 (1)	Feb (2)	Mar (3)	Apr (4)	May (5)	June (6)	July (7)	Aug (8)	Sept (9)	Oct (10)	Nov (11)	Dec (12)	Jan 58 (13)
							Top of Bins						
Maximum	42	46	50	43	49	59	66	66	65	62	54	48	44
Average	39.0	37.8	39.7	41.5	45.2	52.9	58.7	58.9	56.0	55.5	48.8	45.2	40.0
Minimum	35	34	37	38	42	48	54	54	48	51	45	43	39
							Bottom of Bins						
Maximum	41	40	40	41	46	55	61	59	57	57	51	47	43
Average	36.9	35.7	37.6	39.8	43.2	50.0	55.4	55.5	52.7	51.5	46.6	44.0	39.7
Minimum	31	32	35	38	40	45	52	48	46	45	41	40	39
							Center of Bins						
Maximum				39	44	56	55	60	58	55	51	50	46
Average				38.0	41.2	52.2	52.7	57.4	52.4	51.9	47.6	47.0	44.4
Minimum				37	38	46	51	53	49	49	44	45	43

Figure 1

TABLE 2
Percent Culled

Treatment	Storage Time in Months [1]						Average Cull [2]	Over-all Cull [3]
	2	4	6	8	10	12		
Maine Russet Control	8.5	12.7	18.8	6.4	15.8	28.8	13.3	47.7
Maine Russet 14-20,500 rads	55.2	38.4	38.7	35.4	20.0	16.3	46.5	84.2
Maine Russet 28-41,000 rads	67.1	45.0	44.9	33.8	33.1	63.4	58.9	89.6
Maine Katahdin Control	10.9	17.1	16.6	10.4	16.3	45.7	16.1	53.4
Maine Katahdin 14-20,500 rads	68.1	55.0	33.4	66.4	100.0	——	63.2	87.8
Maine Katahdin 28-41,000 rads	59.2	55.3	56.6	43.4	100.0	——	58.1	91.6
Idaho Russet Control	54.3	22.4	21.4	8.8	8.6	21.2	35.0	72.7
Idaho Russet 14-20,500 rads	70.6	39.6	32.2	35.5	17.1	17.0	57.8	88.7
Idaho Russet 28-41,000 rads	82.5	43.8	34.6	39.5	25.4	20.0	73.3	91.6

[1] The percentages for the bimonthly culls are based on the weights of culls removed divided by the weights in storage at the beginning of each culling date.
[2] Based on the total weights of culls removed throughout the storage period divided by the totals of the weights in storage at the beginning of each culling date.
[3] Based on the total weight culled divided by the weight received at the start of the study.

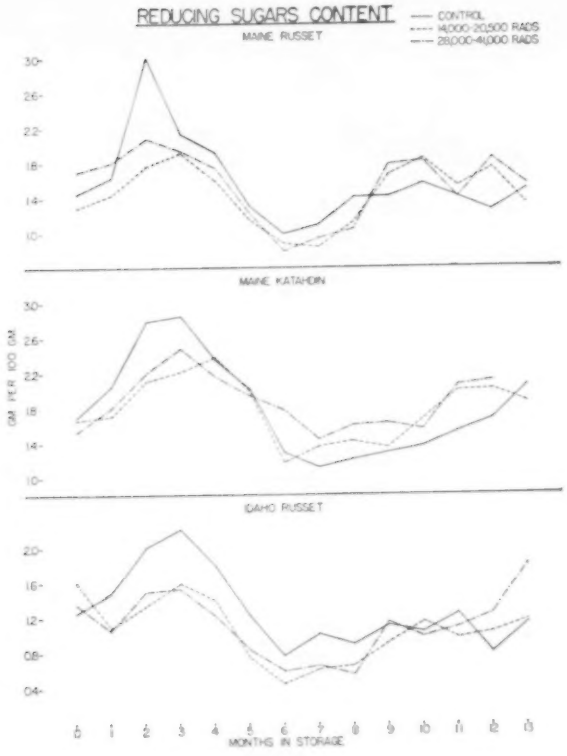

Figure 2

sugars and rise in the starch content. The opposite was true during the colder months. This is obvious when comparing the temperatures of storage in Table 1 with Figures 1, 2, and 3.

The only difference of possible commercial significance between the irradiated tubers and the controls was the higher reducing and total sugars content of the irradiated Maine Katahdins during the last half of the storage period.

Murphy *(10)* reported that the loss of one-third to one-half of the total loss of ascorbic acid in potatoes occurs during the first month of storage. Leichsenring and Norris *(8)* indicated that removing tubers from lower temperatures and storing them at room temperature resulted in an apparent increase in both total and reduced ascorbic acid content.

Both of these phenomena were evident with all three varities used in this study whether they were or were not irradiated as can be seen in Figure 4. There was an initial loss which was followed with the onset of warmer temperatures by a buildup of ascorbic acid. During the latter phase of storage

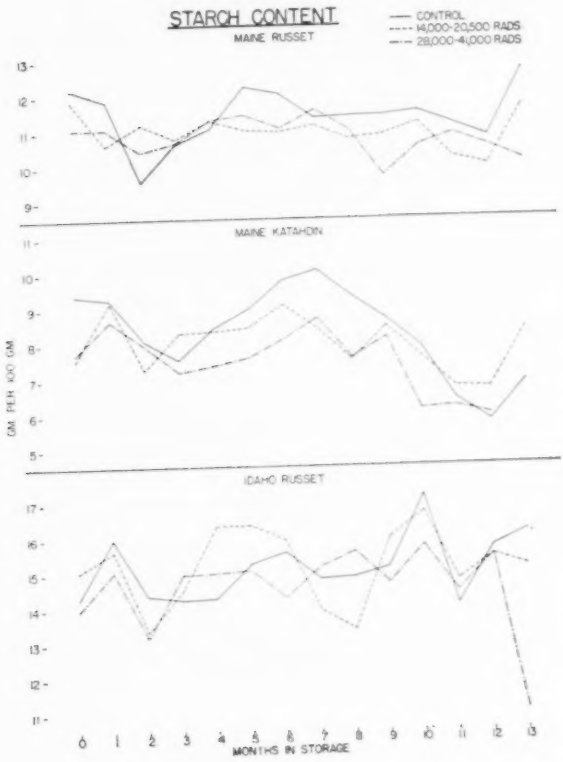

Figure 3

there was another loss which was more apparent in the control than in the irradiated samples.

Throughout the course of this study there did not appear to be any significant differences in the specific gravity between the control and the irradiated samples. In general the controls were slightly higher but the differences were of no commercial significance.

There did not appear to be any commercial significance for the moisture and respiration results.

SUMMARY

Under the conditions of this study the most significant result was the much greater rotting of the irradiated samples when compared with the controls. The lots irradiated at the high dosages had 33.0% more rot than the controls and the low dosage lots had 29.0% more rot than the controls.

There did not appear to be any commercially significant differences in any of the biochemical constituents covered in this study, with the possible exception of the reducing and total sugars values for the Maine Katahdins

towards the last half of the storage period. From the seventh through the twelfth month the controls exhibited lower reducing and total sugars values than their respective irradiated samples. During this period the controls averaged 0.32% lower reducing sugars and 1.22% lower total sugars.

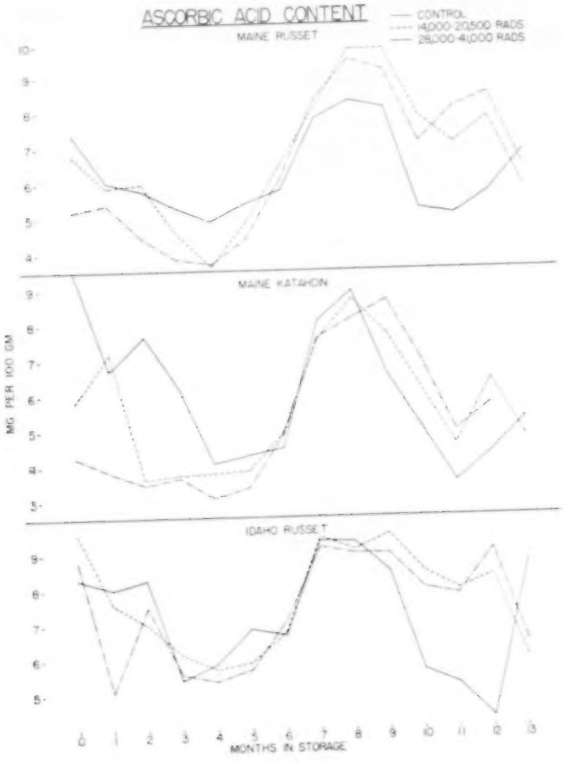

Figure 4

LITERATURE CITED

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EFFECTS OF RADIATION AND OF COOKING ON THE QUALITY OF BABY BEEF LIVER

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This study, was undertaken to determine the effects of radiation and of cooking on baby beef liver. Liver was chosen because it has been reported that organ meats are the least susceptible of meat products to the deleterious side effects of radiation. If radiation could increase the keeping quality of liver without producing undesirable side effects, the benefits would be great, especially since the length of keeping quality of fresh organ meats is less than that of other meats. These meats are also highly nutritious.

Objectives of the investigation were to determine palatability, cooking losses, objective color, moisture, fat, pH and thiamine. Cooking methods were: (a) electric test oven; and (b) electronic oven. Since the use of the electronic oven is increasing both in public eating places and in the home, it is of interest to know the effect of this method of cooking on palatability, cooking losses and cooking time of both unirradiated and irradiated meat.

Reports of the effect of radiation on the qualities of liver are limited. Schweigert *(17)* stated that gamma-irradiated organ meats, such as liver, received higher organoleptic ratings than did fresh, cured or smoked pork products, or beef. Huber, Brasch and Waly *(9)* found that organoleptic and color sensitivity to radiation decreased from beef to lamb to pork to organ meats. They postulated that the greater resistance of organ meats to radiation might be due to the presence of protective substances. They also found that the addition of less than 1% of a homogenate of a cell-free press juice obtained from one of the organ meats minimized the adverse flavor changes in muscle meat.

With regard to odor, Brasch and Huber *(2)* reported that calves' liver had "good" odor after beta radiation with 6 million rep and storage at 37.5° C. for 5, 6 and 12 days. Green, Heindel, Deolalkar and Glenn *(7)*, however, noted a disagreeable odor in beef liver after 1×10^6 to 6×10^6 rep beta radiation at 0° C. Kirn, Urbain, and Czarneski *(11)* observed a decrease in odor scores of from 10 to 8 after radiation with 2×10^6. Doty, Schweigert, Niven and Kraybill *(4)* noted the formation of simple odoriferous sulfur-containing compounds (hydrogen sulfide and methyl mercaptan) and an increase in carbonyls when meat was irradiated at dosages above 1.5×10^6 rep.

Observations on flavor and color have been somewhat divergent. Brasch and Huber *(2)* concluded that irradiated calves' liver had "good" flavor. Kirn *et al. (11)*, however, reported a decrease in flavor scores from 10 to 7.4 after irradiation. Urbain *(19)* suggests that radiated flavor in foods, even though detectable by an expert taster, may go unnoticed by a non-expert panel. As to color, damage to the red pigments, as judged subjectively, in beef liver

following irradiation has been reported by Kirn *et al.* *(11)* and Brasch and Huber *(2)*. Green *et al.* *(7)*, however, found "no noticeable change in the color of irradiated beef liver." Change in the color of beef muscle meat due to radiation has been reported by several laboratories.

Many other interesting observations have been made. Thus, Kirn *et al.* *(11)* reported that beef liver exhibited extensive shrinkage and expression of considerable meat juice after 2×10^6 rep gamma radiation during refrigeration. Brasch and Huber *(2)* reported that calves' liver had a "fair appearance" after 6×10^6 rep beta irradiation and storage at 37.5° C. for 5, 6, and 12 days. Mead *(14)* found that thiamine is highly susceptible to radiation. Effects of radiation on vitamin destruction differ in type and in quantity from food to food. Increased destruction of thiamine with increased dosage has been found with most foods. Goldblith *(6)* noted that labile nutrients in foods are "protected" remarkably from massive destruction during radiation by the presence of complex materials, either naturally occurring protective substances or naturally occurring radio-resistant compounds. No reports on the effect of radiation on vitamins in liver were found in the literature.

EXPERIMENTAL PROCEDURE

History and radiation treatment. Frozen whole baby beef livers were purchased on the open market. Each liver while still frozen was sliced on an electric meat slicer to a thickness of one-half inch.

From each slice of liver a rectangular-shaped piece, 4½ in. by 2½ in., weighing approximately 85 g. was cut. Separable fat, membrane, and gristle were removed. Slices to be cooked were sealed into moisture-vapor-resistant cellophane and divided into 3 lots according to the pretreatment to be given: (a) control; (b) 3 million rep beta radiated; and (c) 6 million rep beta radiated.

Smaller pieces of liver and the outside pieces of larger slices were sealed in cellophane, labelled, frozen, and stored for use in color determinations and chemical analysis of the raw meat.

The 3 lots were frozen in a home-type chest freezer set at −20° F. The control samples remained there until they were studied. Two lots were packed in dry ice, and shipped by express to Cambridge, Mass., where they were irradiated[a] on each broad side while still frozen.

The frozen radiated liver was then repacked in dry ice and returned to Ithaca and stored in a home freezer (−20° F.) until tested (within 4 weeks).

METHODS

Sampling for analysis. *Raw Meat:* One slice of frozen liver from each of the 3 lots was sliced through the center horizontally. One of the half-slices was used for objective color determination. The other half-slice and another whole slice were ground twice, with mixing after each grinding, in preparation for chemical analysis. Slicing and grinding were started while the liver was frozen to minimize drip losses.

Cooked meat: After samples for color and palatability tests were obtained, the remainder of the cooked liver was ground, mixed well, and aliquots taken for chemical analysis.

Cooking methods. *General methods:* Each test was repeated 4 times. All of the liver was cooked from the solidly frozen state.

A cooking lot consisted of 4 slices of liver from each of the 3 pretreatments. Since one method of cooking was the electronic oven in which metal pans are not suitable, glass baking pans, 8¼ in. square and 2 in. deep, were used for both cooking methods.

The liver was cooked a predetermined time by both cooking methods. Preliminary

[a] Irradiation by means of a Van de Graff accelerator.

tests indicated that the use of thermometers or thermocouples, to determine internal temperature of the liver, was not practical, since the thickness of the samples was not sufficient to hold either the thermometers or thermocouples firmly in the meat.

Conventional method: No directions for baking liver in the oven without the addition of some liquid were found in the literature. Therefore, the cooking method used was a modification of that for broiling liver: lower temperature for a longer time. Baking made possible a greater control and uniformity of heat than broiling. A well-insulated electric test oven [b] was preheated to a temperature of 400° F. Three baking dishes, each containing liver of a single treatment were placed on racks on the hearth of this oven and cooked simultaneously for 25 minutes. The hearth of the oven was rotated during cooking.

Electronic oven [c]: Each lot was cooked successively according to a statistical design:

Day of Cooking	Order of Cooking in Electronic Oven		
	1	2	3
1	C	B	A
2	A	B	C
3	C	A	B
4	A	C	B

A = Control
B = 3 million rep
C = 6 million rep

The electronic oven was set at "high". The baking pans were covered with cellophane throughout cooking to avoid excessive drying and losses due to spattering. After 2½ min. of cooking, the slices were turned over and were also rotated so that the sides that had been toward the outside of the pan were toward the center. The liver was then cooked 2½ min. longer, a total of 5 minutes. Preliminary tests had shown that if the slices were not turned midway through cooking in the electronic oven, outside edges were overcooked, and center portions were underdone and pinkish in color.

Palatability testing. Cooked samples to be used for palatability testing were brushed with a weighed quantity of melted butter and sprinkled with a weighed quantity of salt on each side just before tasting. This treatment was not given to the slices to be used for chemical analysis.

Liver samples were given organoleptic rating by a panel of 6 experienced judges from the Food and Nutrition, Institution Management, Animal Husbandry and Hotel School faculties. Judges scored separately 2 sets of 3 samples each on each cooking day. Each set consisted of a control, a 3 million rep, and a 6 million rep sample cooked by each method. Thus all of the variables (3 pretreatments and 2 cooking methods) were compared at one testing.

Each judge was given one-fourth of a slice of liver. To avoid the possibility of one judge consistently receiving an inside or an outside section, judges were assigned inner (I) or outer (O) sections according to a statistical design.

Cooking Day	Judge					
	1	2	3	4	5	6
1	I*	I	I	O	O	O**
2	O	O	O	I	I	I
3	I	O	O	O	I	I
4	O	I	I	I	O	O

* I = Inner ** O = Outer

The score card is given in Table 1. Judges checked descriptive terms to which arbitrary values were given in interpreting differences. Maximum possible score was 5. Judges also stated whether they considered samples acceptable for civilian use and/or

[b] DESPATCH oven, a well-insulated electric test oven with a rotating hearth.
[c] RADARANGE, Raytheon Manufacturing Company, Waltham, Massachusetts.

The order of tasting samples also followed a randomized design:

Day of Cooking	Type of Cooking	Order of Tasting		
1	Conventional	C	A	B
	Electronic	C	B	A
2	Conventional	B	C	A
	Electronic	A	B	C
3	Conventional	A	C	B
	Electronic	C	A	B
4	Conventional	C	B	A
	Electronic	A	C	B

A = Control
B = 3 million rep
C = 6 million rep

military use. Acceptability was calculated as per cent of positive responses. Order of preference was also given.

Cooking data. The cooking record included (a) weight of raw and cooked liver; (b) cooking weight loss; (c) drip loss; and (d) evaporation loss.

Objective color determinations. The Hunter Color and Color Difference Meter was used to measure the "Rd," "a" and "b" values of the inside cross section of the slices of liver. Color was reported as the a/b ratio, or hue. A standard color plate having values of Rd 2.4; "a" 18.2 and "b" 8.0 was used in balancing the color meter.

Moisture. Five grams of ground liver was dried in a vacuum oven at 95° under a 26 to 28 in. vacuum for 7 hours.

Fat. Fat was extracted by the method of Watts and Peng *(21)* with slight modification. Twenty grams of ground liver was used for each analysis.

Thiamine. Sixty grams of raw and 50 g. of cooked, ground liver were used for each analysis. Grinding of raw liver was started while it was still frozen. Kotschevor *(12)* reported thawing of sliced unirradiated calves liver at room temperature for 4 hrs., or at 40° F. for 20 hrs., resulted in a loss of thiamine. He found an appreciable amount of thiamine present in the drip.

Thiamine was extracted from the ground liver by the method of Watts, Peng and Esselbaugh *(22)* with slight modification, and was measured by the thiochrome method of Conner and Straub *(3)*. Results are reported on both the moist, fat basis and the dry, fat-free basis.

Statistical design and analysis. Analysis of variance according to Snedecor *(18)* was applied to the data. Four replicates of each study were made.

RESULTS AND DISCUSSION

Subjective Observations

Raw meat. The appearance of the irradiated liver was not noticeably different from that of the control. Green *et al. (7)* also found no noticeable change in color of irradiated beef liver when judged subjectively. Kirn *et al. (11)*, judging subjectively, and Brasch and Huber *(2)*, however, observed damage to the red pigments of liver by radiation. Kirn *et al. (11)* reported that beef liver, gamma irradiated with 2×10^6 rep during refrigeration, exhibited extensive shrinkage and expression of meat juice.

Cooked meat. Differences in texture were noted in cutting the slices for palatability testing and in grinding the meat for analysis. With increased radiation dose, the meat became progressively more crumbly. In the 6 million rep lot it was almost impossible to distinguish the small bits of gristle present from the liver tissue itself. Radiation had apparently broken the cell structure.

TABLE 1

Palatability score card for baby beef liver

Judges Name_____ Date_____

Note: Check (V) the descriptive term you think applies. Take a separate piece of meat, cutting each about the same size, in judging each quality, concentrating on one quality at a time. Give your impression of juiciness after 2 chews.

			Sample Number			COMMENTS
			A	B	C	
ODOR	Natural	(5)				
	Off-odor: Weak	(4)				
	Moderate	(3)				
	Strong	(2)				
	Describe Objectionable Odor					
FLAVOR	Natural	(5)				
	Off-flavor: Weak	(4)				
	Moderate	(3)				
	Strong	(2)				
	Describe Objectionable Flavor					
COLOR OF CUT SURFACE	Natural	(5)				
	Off-color: Too light	(3)				
	Too dark	(3)				
	Describe Objectionable Color					
TEXTURE OF CUT SURFACE	Compact (Natural)	(5)				
	Not Compact or Porous	(3)				
JUICINESS	Natural	(5)				
	Too dry	(3)				
	Too moist	(3)				
TENDERNESS	Natural	(5)				
	Too tender	(3)				
	Too tough	(3)				
ACCEPTABILITY (write yes or no)	Under civilian conditions					
	Texture					
	Odor					
	Flavor					
	Color					
	Juiciness					
	Tenderness					
	Under extreme military conditions					
	Texture					
	Odor					
	Flavor					

	Color				
	Juiciness				
	Tenderness				
	Do you think you could become accustomed to this flavor and accept it? (Yes or no)				

ORDER OF PREFERENCE:

Morgan *(15)* states that if a protein is radiated wet there seems to be a tendency for it to increase in viscosity almost to the point of gel formation probably due to cross linkage. Softening of the tissues of certain vegetables and fruits by irradiation has been reported by Kertesz *et al. (10)* and Glegg *et al. (5)*.

Both the amount of coagulum and cooking drip decreased with radiation of the liver.

Cooking method. Liver cooked in the electric test oven was a glazed mahogany color on the outside. Little coagulum and no fluid remained in the pans after cooking.

Although samples cooked in the electronic oven were not browned, the surface was an even, dark tannish color. This method resulted in a considerable amount of coagulum which was the same uniform color as that of the meat, and also in an appreciable amount of relatively clear, tan liquid. The larger amount of drip present was no doubt due to less evaporation as a result of the shorter cooking time.

Palatability testing

Palatability scores [4] are presented in Table 2, the significance of differences in Table 3.

Odor. *Radiation:* The mean odor score for the control, 4.7, was significantly higher than for the irradiated samples, 3.7 and 3.4. This trend agrees with that reported by Green *et al. (7)* and Kirn *et al. (11)* but not with that reported by Brasch and Huber *(2)*. No statistically significant difference occurred between the radiated lots (Table 3).

Judges described the odor of the radiated samples as: "not characteristic but not objectionable," "protein decomposition," "rancid" or "stale." One judge, however, who had been working with radiated meats for some time consistently judged the radiated samples as "natural" in odor. This may indicate that it is possible to become accustomed to the radiated odor and accept it.

Cooking method: Differences in odor scores due to cooking method (4.1 and 3.7) were not statistically significant.

Flavor. *Radiation:* Mean flavor scores were significantly higher for control, 4.7, than for radiated samples, 3.6 and 3.1. The difference, however, was not as great as that found for ground pork in another study in this

[4] It is possible that the palatability scores of the control lot of liver might have been lower if it had been subjected to transportation to and from Cambridge. The aim, however, in this study was to make practical comparisons between the quality of irradiated liver and that which had not been subjected to hazards.

TABLE 2

Effect of irradiation on mean palatability scores [1] of baby beef liver cooked conventionally and electronically

Quality factor	Pretreatment	Cooking methods		Pretreatment means
		Electric oven	Electronic oven	
Odor	Control	4.8	4.6	4.7
	3 million rep	3.8	3.6	3.7
	6 million rep	3.8	3.0	3.4
	Means	4.1	3.7	
Flavor	Control	4.8	4.8	4.7
	3 million rep	3.8	3.5	3.6
	6 million rep	3.2	3.0	3.1
	Means	3.9	3.8	
Color	Control	5.0	5.0	5.0
	3 million rep	4.8	4.8	4.8
	6 million rep	4.9	4.6	4.7
	Means	4.9	4.8	
Texture	Control	4.8	4.9	4.9
	3 million rep	4.3	4.0	4.2
	6 million rep	4.1	3.7	3.9
	Means	4.4	4.2	
Juiciness	Control	4.8	4.5	4.6
	3 million rep	3.9	3.7	3.8
	6 million rep	3.9	3.4	3.7
	Means	4.2	3.9	
Tenderness	Control	4.7	4.5	4.6
	3 million rep	3.6	3.9	3.8
	6 million rep	3.4	3.2	3.3
	Means	3.9	3.9	

[1] Maximum possible score 5.

laboratory *(13)*. Terms used in describing flavor of radiated liver were similar to those for odor. Kirn *et al. (11)* also reported lower flavor scores for radiated beef liver. Brasch and Huber *(2)*, however, found that irradiated calf liver had a "good" flavor.

Cooking method: Differences in flavor scores due to cooking method (3.9 and 3.8) were not statistically significant.

Color. *Radiation:* The cut surface of the cooked control meat received a significantly higher mean score, 5.0, than did that of either of the radiated samples, 4.8 and 4.7. Since the difference was only 0.2 or 0.3 unit, its significance is questionable. Furthermore, 4 of the judges gave all of the radiated liver "natural" color ratings on every testing day. Two of the judges, however, rated the color of the radiated samples consistently lower than that of the control.

Cooking method: Differences in color of cut surface due to cooking method were not statistically significant, 4.9 and 4.8.

Texture. *Radiation:* Mean texture score for the control, 4.9, was signif-

TABLE 3

Mean squares and significance of palatability scores of irradiated baby beef liver
Pretreatment: 0 = Control; 3 = 3 million rep lot; 6 = 6 million rep lot

Source	d.f.	Palatability factors					
		Odor	Flavor	Color	Texture	Juiciness	Tenderness
Replicates	3	0.10153	0.20778	0.04000	0.16264	0.29931 *	0.56444 *
Cooking Method	1	1.00042	0.24000	0.04167	0.26042	0.57042 *	0.00167
Error (a)	3	0.33375	0.15667	0.01500	0.21264	0.02375	0.05500
Pretreatment	2	3.78042 **	5.72042 **	0.17167 **	2.03625 **	2.27625 **	3.59292 **
0 vs. 3 + 6	1	7.28521 **	10.17521 **	0.33333 **	3.79688 **	4.50188 **	6.23521 **
3 vs. 6	1	0.27563	1.26563 **	0.01000	0.27563	0.05063	0.95063 *
Pretreatment × Cooking Method	2	0.23042	0.02375	0.04167	0.14542	0.05792	0.15042
0 vs. 3 + 6 × Cooking Method	1	0.13021	0.01688	0.02083	0.28521 *	0.01021	0.07521
3 vs. 6 × Cooking Method	1	0.33063	0.03063	0.06250	0.00563	0.10563	0.22563
Error (b)	12	0.08264	0.08597	0.01500	0.05806	0.12819	0.12556
ADDITIONAL COMPARISONS [1]							
0 vs. 3	1	1.00000 **	1.21000 **	0.04000 *	0.49000 **	0.64000 **	0.64000 **
0 vs. 6	1	1.69000 **	2.56000 **	0.09000 **	1.00000 **	1.69000 **	10.89000 **

* Signifiant, 5% level.
** Highly significant, 1% level.
[1] Dunnett, C. W. A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.*, 50:1096-1121. 1955.

icantly higher than for radiated, 4.2 and 3.9. No statistically significant difference occurred between the 2 radiated lots. Judges described the texture of the radiated samples as slightly porous.

Cooking method: Differences in mean texture scores due to cooking method were not statistically significant, 4.4 and 4.2.

Juiciness. *Radiation:* Control meat received a significantly higher mean score, 4.6, than the radiated samples, 3.8 and 3.7. Radiated samples were considered "too dry."

Cooking method: Liver cooked conventionally received significantly higher juiciness scores, 4.2, than that cooked electronically, 3.9. This difference of only 0.3 unit is probably of no practical significance. Again, electronically cooked samples received lower mean scores because they were considered "too dry".

Tenderness. *Radiation:* Mean tenderness score for the control, 4.6, was significantly higher than scores for radiated samples, 3.8 and 3.3. Radiated meat was considered "too tender." The 6 million rep samples were described as powdery, crumbly, or too mushy.

Cooking method: Experimental means for tenderness scores due to cooking method were the same, 3.9.

Civilian and military acceptability. In estimating acceptability of flavor to civilians, judges responded in the affirmative to the control 89.6% of the time, to the 3 million rep lot 50% of the time, and to the 6 million rep lot 41.7% of the time. In estimating flavor acceptability to the military, affirmative answers were: control 95.9%, 3 million rep lot 68.8% and 6 million rep lot 62.5% (Table 4). Thus there was more difference in acceptability between the control and the irradiated meat than between the two irradiated lots.

Perhaps the acceptability scores of the radiated meat on all quality factors would have been higher if the control had not been presented at the same time, and also if the meat had been included in a meal with other foods.

Order of preference. Order of preference for both cooking methods was: first choice — control; second — 3 million rep; and third — 6 million rep lot. The control was ranked 1st, 42 times, 2nd, 4 times and 3rd, 10 times; the 3×10^6 rep lot, 1st twice, 2nd, 45 times and 3rd, 10 times; the 6×10^6 rep lot, 1st twice, 2nd, 11 times and 3rd, 33 times.

Cooking weight losses. *Radiation:* Mean cooking weight losses of the control lot, 21.4%, were significantly higher than for the radiated samples, 18.8 and 17.0% (Tables 5 and 6). No reason for this finding is known. Cooking time for the 3 lots was the same.

Cooking method: Differences in weight loss due to cooking method were the same, 19.0 and 19.1%.

Cooking drip losses. *Radiation:* Drip loss of the control, 4.7%, was significantly greater than that of the radiated lots.

Reduction in drip loss in cooking radiated samples is similar to findings of Lim, Yen and Fenton *(13)* who noted reduced cooking drip in ground pork patties following radiation.

Cooking method: A larger amount of drip, 5.1%, was found in electronic cooking than in conventional cooking, 0.5%, probably chiefly because of the shorter cooking time which would allow less evaporation of drip.

Statistically significant interaction occurred between pretreatment and

TABLE 4

Acceptability [1] for civilian and military use of irradiated baby beef liver cooked by two methods

Quality Factor	Pretreatment	Civilian acceptability			Military acceptability		
		Electric oven	Electronic oven	Pretreatment means	Electric oven	Electronic oven	Pretreatment means
		%	%	%	%	%	%
Odor	Control	95.8	95.8	95.8	100.0	95.8	97.9
	3 million rep	70.8	54.2	62.5	83.3	79.1	81.2
	6 million rep	62.5	41.7	52.1	91.7	66.7	79.2
	Means	76.4	63.9		91.7	80.5	
Flavor	Control	91.7	87.5	89.6	100.0	91.7	95.9
	3 million rep	58.3	41.7	50.0	66.7	70.8	68.8
	6 million rep	50.0	33.3	41.7	70.8	54.2	62.5
	Means	66.7	54.2		79.2	72.2	
Color	Control	100.0	100.0	100.0	100.0	100.0	100.0
	3 million rep	87.5	79.2	83.4	95.8	95.8	95.8
	6 million rep	83.3	79.2	81.3	100.0	100.0	100.0
	Means	90.3	86.1		98.6	98.6	
Texture	Control	88.8	100.0	94.4	88.9	100.0	94.5
	3 million rep	61.1	66.7	63.9	94.4	94.4	94.4
	6 million rep	38.8	38.9	38.9	83.3	77.8	80.6
	Means	62.9	68.5		88.9	90.7	
Juiciness	Control	95.8	91.7	93.8	100.0	100.0	100.0
	3 million rep	50.0	50.0	50.0	79.2	83.3	81.3
	6 million rep	50.0	37.5	43.8	79.2	62.5	70.9
	Means	65.3	59.7		86.1	81.9	
Tenderness	Control	95.8	95.8	95.8	100.0	95.8	97.9
	3 million rep	54.2	50.0	52.1	75.0	91.7	83.4
	6 million rep	45.8	33.3	39.6	75.0	66.7	70.9
	Means	65.3	59.7		83.3	84.7	

[1] Per cent positive responses.

TABLE 5

Effect of irradiation on mean losses during conventional and electronic cooking of baby beef liver

Cooking loss	Pretreatment	Method of cooking		Pretreatment mean
		Electric oven	Electronic oven	
	rep	%	%	%
Weight	Control	20.4	22.4	21.4
	3 million	19.0	18.6	18.8
	6 million	17.5	16.4	17.0
	Means	19.0	19.1	
Drip	Control	1.0	8.4	4.7
	3 million	0.5	4.5	2.5
	6 million	0.0	2.4	1.2
	Means	0.5	5.1	
Evaporation	Control	19.3	14.1	16.7
	3 million	18.5	14.2	16.4
	6 million	17.5	14.0	15.8
	Means	18.4	14.1	

TABLE 6

Mean squares and significance of cooking losses of irradiated baby beef liver
Pretreatment: 0 = Control; 3 = 3 million rep lot; 6 = 6 million rep lot.

Source	d.f.	Cooking losses		
		Percent weight loss	Percent total drip loss	% evaporation loss
Replicates	3	5.22486	2.31084	11.34153
Cooking Method	1	0.26042	125.08101 **	113.97042
Error (a)	3	19.00153	1.15345	28.79819
Pretreatment	2	40.19042 **	24.74976 **	1.98042
0 vs. 3 + 6	1	66.50521 **	43.11125 **	2.52083
3 vs. 6	1	13.87563 *	6.38826 *	1.44000
Pretreatment Cooking Method	2	5.35042	12.57850 **	1.58042
0 vs. 3 + 6 × Cooking Method	1	10.17521	22.83900 **	2.52083
3 vs. 6 × Cooking Method	1	0.52563	2.31801	0.64000
Error (b)	12	2.79486	1.04769	3.54653
ADDITIONAL COMPARISONS [1]				
0 vs. 3	1	6.76000 *	4.84000 **	0.90000
0 vs. 6	1	19.36000 **	12.25000 **	8.10000 *

* Significant, 5% level.
** Highly significant, 1% level.
[1] Dunnett, C. W. A multiple comparison procedure for comparing treatments with a control. *J. Amer. Statis. Assoc.* 50:1096. 1955.

cooking method. Difference in drip loss between the control and the radiated samples was greater in the electronic oven than in the conventional oven.

Evaporation loss. *Radiation.* Evaporation loss for the control, 16.7%, was significantly larger than that for the 6 million rep lot, 15.8%, but not higher than that for the 3 million rep lot, 16.4%. The differences are probably too small to be of any practical importance.

Cooking method: Differences in experimental means for evaporation loss

in the 2 cooking methods were not statistically significant, 18.4% and 14.1% (Tables 5 and 6).

Objective Color

Raw meat. *Radiation:* Statistically significant difference was found between the a/b ratios of the cut surface of the control, 1.44, and radiated samples, 1.66 and 1.75. Differences between the radiated lots were not significant (Tables 7, 9 and 10). When the meat was judged subjectively no difference in color was noticed.

Cooked meat. *Radiation:* Differences between experimental means for a/b color ratio of cut surface of cooked liver were not significant, 1.05, 1.09 and 1.06.

Cooking evidently obliterated the differences in objective color values obtained in the raw meat.

Cooking method: Differences between experimental means for a/b color ratio for the 2 cooking methods were not significant, 1.11 and 1.03.

Moisture Content

Radiation. *Raw Liver:* Differences in mean moisture content in the 3 lots of liver were not significant: 70.2 to 70.7% (Tables 7 and 10). Scheid, Bennett and Schweigert *(16)* reported the moisture content of unirradiated liver as 69.2 to 73.4%.

Cooked Liver: After cooking, the control lot and the 3×10^6 rep lot had similar moisture content, 57.3 and 57.4%. The 6×10^6 rep lot had significantly higher moisture content, 58.5% (Tables 6, 10 and 11). These small differences probably have no practical importance. It has been suggested, however, that higher dosages of radiation release bound water in muscle meat.

Cooking. Although liver cooked conventionally had a statistically significant higher moisture content, 58.0%, than did that cooked electronically, 57.4%, the difference of only 0.6% probably has no practical significance.

Statistically significant interaction in moisture content of the control and the radiated lot was found between cooking methods.

Fat Content

Radiation. *Raw Liver:* No significant difference in mean fat content was found, 1.3 to 1.7% (Tables 7, 9 and 10). The fat content was lower than that reported in a Food Composition Table, approximately 5% *(10)*. This lower value was probably at least partially due to the removal of separable fat from the liver prior to packaging.

Cooked Liver: No significant difference was found in fat content of the control, 6.3%, or either of the irradiated lots, 6.4 and 6.1%. The slight difference in the 2 radiated lots was probably of no practical significance.

Cooking. Differences in fat content between liver cooked by the 2 methods were not significant, 6.3 and 6.2%.

pH Value

Mean pH values of the 3 lots of liver both before radiation, and before and after the 2 methods of cooking were consistent, 6.2.

TABLE 7

Effect of radiation on mean color, moisture, fat, and thiamine content of raw and cooked liver

Radiation	a/b Ratio				Moisture			Fat			Thiamine					
											Moist, fat basis			Dry, fat-free basis		
	Raw	Cooked Conventionally	Raw	Cooked electronically	Raw	Cooked		Raw	Cooked		Raw	Cooked		Raw	Cooked	
						Conventional	Electronic		Conventional	Electronic		Conventional	Electronic		Conventional	Electronic
rep					%	%	%	%	%	%	*mg/g*	*mg/g*	*mg/g*	*mg/g*	*mg/g*	*mg/g*
Control	1.47	1.03	1.41	1.07	70.2	58.2	56.4	1.3	6.4	6.3	0.205	0.259	0.241	0.719	0.738	0.653
3×10^6	1.73	1.19	1.59	1.00	70.5	57.4	57.3	1.4	6.4	6.4	0.166	0.194	0.187	0.595	0.546	0.522
6×10^6	1.86	1.11	1.62	1.02	70.7	58.4	58.6	1.7	6.2	6.1	0.122	0.146	0.155	0.445	0.422	0.445

TABLE 9

Mean squares and significance of thiamine content [1] and retention, moisture and fat content, and pH value of raw irradiated baby beef liver

Source of Variance	d.f.	Mean squares				
		Thiamine content	Thiamine retention	Moisture content	Fat content	pH value
Replicates	3	0.0236 **	68.6746 **	29.42 **	0.47	0.00
Pretreatment	2	0.0752 **	905.2513 **	0.46	0.16	0.00
Control vs. Irradiated	1	0.1056 **		0.65	0.22	0.00
3×10^6 rep vs. 6×10^6 rep	1	0.0447 **	905.2513 **	0.28	0.10	0.00
Error	6	0.0011	5.0823	1.83	0.16	0.01

[1] Dry, fat-free basis.
** Significant, 1% level.

TABLE 10

Mean squares and significance of thiamine content,[1] thiamine retention,[2] moisture and fat content, pH and a/b values of cooked irradiated baby beef liver

Source of variance	d.f.	Mean squares											
		Thiamine content		Thiamine retention		Moisture content		Fat content		pH value		a/b ratio	
Replicates	3	0.0523		49.5155		154.2971 **		0.5122		0.0093		0.06549*	
Cooking Method	1	0.0049		0.2400		1.6538 *		0.0267		0.0004		0.03760	
Error (a)	3	0.0136		144.6457		0.0949		0.1656		0.0160		0.00487	
Pretreatment	2	0.1397 **		194.3902		3.5379 *		0.1429		0.0004		0.00395	
Control vs. irradiated	1		0.2393 **		22.5365		2.1252		0.06002		0.00		0.00460
3×10^6 rep vs. 6×10^6 rep	1		0.0401 **		366.2438 *		4.9506 *		0.2256 *		0.00		0.00331
Pretreatment × cooking method	2	0.0058 *		151.7761		2.2888		0.0079		0.0004		0.02838	
Control vs. irradiated × Cooking method	1		0.0094 *		219.6924		4.5019 *		0.0002		0.00		0.04625
3×10^6 rep vs. 6×10^6 rep × Cooking method	1		0.0022		83.8597		0.0756		0.0156		0.00		0.01051
Error (b)	12	0.0012		65.9575		0.7756		0.0443		0.0010		0.01256	

[1] Dry, fat-free basis.
[2] Effect of cooking.
* Significant, 5% level.
** Significant, 1% level.

TABLE 11

Mean squares and significance of mean differences between pairs [1] in thiamine content,[2] thiamine retention,[3,4] moisture, and fat content, pH and a/b values of raw and cooked irradiated baby beef liver

Source of Variance	d.f.	Mean squares									
		Thiamine content		Thiamine retention		Moisture content		Fat content		pH value		a/b ratio	
		Raw	Cooked	Raw [3]	Cooked [4]	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
Control vs. 3×10^6 rep	1	0.015 **	0.026 **	364.810 **	7.290	0.090	0.010	0.010	0.010	0.000	0.000	0.04840	0.00160
Control vs. 6×10^6 rep	1	0.075 **	0.069 **	1632.160 **	46.610	0.250	1.440 *	0.160	0.040	0.000	0.000	0.09610	0.00010

[1] Dunnett, C. W. A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* **50**, 1096 (1955).
[2] Dry, fat-free basis.
[3] Effect of irradiation.
[4] Effect of cooking.
* Significant, 5% level.
** Significant, 1% level.

Thiamine Content and Retention

Radiation. *Raw Liver:* As anticipated, thiamine content decreased significantly with increased radiation (Tables 1, 9 and 11). Ziporin, Kraybill and Thach *(23)* report that the degradation of thiamine occurs by the splitting of the molecule into pyrimidine and thiazole moieties and that the thiazole undergoes further degradative changes at higher doses of irradiation.

Mean thiamine contents of raw liver on both the moist, fat basis and on the dry, fat-free basis are presented in Table 7. Scheid *et. al. (16)* found the thiamine content of liver on a fresh basis to be 0.18 to 0.27 mg. per 100 g. The 0.205 mg. content of the control lot in this study was within this range.

Difference in mean retention of thiamine after the 2 levels of radiation was highly significant: 80.9%, and 59.6% (Tables 8, 9 and 10). Nothing was

TABLE 8

Effect of radiation and cooking on retention of thiamine in baby beef liver

Treatment	Thiamine retention		
	Control	3×10^6	6×10^6
	%	%	%
Irradiation only	100	80.9	59.6
Cooking only			
Conventional	100.2	93.3	98.3
Electronic	91.4	92.8	107.0

found in the literature on the effect of radiation on the vitamins in liver. Groninger *et al. (8)*, however, reported the retention of thiamine in oxygen-packed, gamma-irradiated beef as 76% after 1×10^6 rep; 16% after 3×10^6 rep and 6% after 10×10^6 rep. Alexander, Day, Sauberlich and Salmon *(1)* stated that, at a dose of 3×10^6 rep, thiamine content of beef was decreased by 60 to 67%.

Cooked liver: Decrease in thiamine content of raw liver with increase in radiation carried through to the cooked liver (Table 7). Differences in thiamine content of the 3 lots of liver on the dry, fat-free basis were highly significant (Tables 9 and 10). Mean thiamine content on the moist, fat basis of each lot cooked by the two methods was: 0.250 mg., 0.190 and 0.150 mg. per 100 g. On the dry, fat-free basis, mean thiamine content of each lot was 0.695 mg., 0.534 mg., and 0.433 mg. per 100 g.

Cooking. Differences in thiamine content after the 2 methods of cooking were not statistically significant (Tables 9 and 10).

Little loss of thiamine occurred during the cooking of these thin slices of liver. Differences in mean thiamine retention during cooking of the control and the irradiated liver were also not significant.

Radiation plus cooking. Mean retentions of thiamine after irradiation plus cooking of the 3×10^6 rep lots of liver were about the same for the 2 methods of cooking.

SUMMARY

A study was made of the effect of beta radiation on palatability, cooking losses, objective color, moisture, fat, pH and thiamine of baby beef liver. Pretreatments were: control, 3 million rep and 6 million rep radiation. The meat was irradiated in the frozen state.

Cooking methods used were conventional (electric test oven) and electronic oven. Slices of baby beef liver were cooked from the hard-frozen state for 25 min. in the electric test oven, and 5 min. in the electronic oven.

Irradiation: Radiation resulted in decreased palatability scores for odor, flavor, texture, juiciness, and tenderness; decreased preference rank; decreased cooking weight loss and cooking drip loss. Decrease in palatability qualities, particularly those due to oxidation, might have been less if the meat had been packed in an inert gas. Irradiation resulted in a thiamine retention of 80.9% in the 3×10^6 rep lot and 59.6% in the 6×10^6 lot.

The a/b color ratio of the cut surface of the raw meat increased. Moisture and fat of the raw liver were not significantly affected by radiation, nor were evaporation losses during cooking, or the a/b color ratio of the cut surface of the cooked meat, or the pH.

Cooking: Electronic cooking resulted in slightly lower juiciness scores and higher cooking drip losses than did cooking in the electric test oven for all 3 lots. Odor, flavor, color, texture and tenderness palatability scores, cooking weight loss, evaporation loss, a/b color ratios of cut surface, pH, and losses of moisture, fat, and thiamine were not significantly different in the liver cooked electronically and conventionally.

Statistically significant interaction between pretreatment and cooking method was observed in texture and in cooking drip loss. The electronic oven resulted in greater difference between control and radiated samp'es than did the electric oven.

Electronic cooking of one lot (4 slices) required one-fifth the time of the electric test oven cooking of 3 lots (12 slices) of liver.

Cooking these thin slices of liver resulted in little further loss of thiamine.

More studies are needed since work on muscle meats indicates that packing the liver in an inert gas might protect it from deleterious oxidative side reactions. Allowing the frozen liver to stand exposed to the atmosphere at room temperature before it is cooked might also result in a decrease in the undesirable flavor.

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COMPOSITION OF TEN PROCESSING AND TABLE VARIETIES OF VIRGINIA APPLES*

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This study was undertaken to determine the important chemical constituents of 10 varieties of apples grown in ten apple-producing centers located in Virginia. Certain other physical and chemical characteristics of apples were also studied. Seven of the 10 varieties analyzed in the course of the work reported here are important processing varieties: York Imperial, Stayman, Golden Delicious, Grimes Golden, Jonathan, Rome Beauty, and Albemarle Pippin (Yellow Newton). The other three are table apples: Delicious, Winesap, and Lowry. Data on the chemical composition of apples are useful not only for nutritionists but also for processors.

A considerable amount of literature exists on the chemical constituents of apples. The first chemical analysis ever reported on the whole fruit of American apples was probably the one published in 1887 by Richards *(29)*. Other early studies were made by Browne *(7)* in 1900, Alwood *(1, 2)* and by Alwood, Davidson, and Moncure *(3, 4)* between 1902 and 1904. In 1904 Van Slyke *(34)* published analyses of 75 varieties of apples and 10 varieties of crab-apples. Gore *(19)* in 1908 reported on the composition of the fresh juices of 11 apple varieties.

Jones and Colver *(24)* in 1912, published several extensive analytical studies on the composition of all the important commercial varieties being grown in Idaho at that time. In the same year, Shaw *(32)* reported the results of his work with 21 varieties of apples grown in many localities of the United States. His work included the determination of several constituents as well as the inter-relationships between the conditions of soil, climate, and culture on the composition and eating quality of apples.

In 1925, Frisk *(18)* compared the nutritive value of 5 varieties of Iowa winter apples. In 1928 Caldwell *(8, 9, 10)* published the results of determinations of several constituents on a very large number of American and French varieties of Virginia-grown apples. Cooking and eating apples were included in a 1928 study made by Lampitt and Hughes *(25)* in an article on the general composition of various fruits. In that year also, Fellers *(17)* published results of analytical work done on the chief Massachusetts commercial apple varieties. St. John and Morris *(30)* in 1929 showed that the level of sugars and other constituents of Jonathan apples varies from year to year.

In 1937 Todhunter *(33)* assembled all the data on the nutritive value of apples available up to that time. The results of a 5-year study on acidity and pH of 13 varieties of New York State apples were reported in 1931 by Pfund *(28)*. In 1947, Esselen *et al. (16)* summed up the information available on

* A portion of the data presented in this paper was taken from a thesis presented by Paul J. Fellers to the Graduate School of Virginia Polytechnic Institute in partial fulfillment for the requirements of the degree of Master of Science.

the composition and nutritive value of apples. In 1950, Smock and Neubert *(32)* published a book containing information on the apple fruit after harvest, including its composition.

EXPERIMENTAL

Source of material. All apple fruit used in the work reported here were from the 1955 Virginia apple harvest. The various samples were collected from cold and common storages located in the main producing areas of the state. Samples of the processing varieties in most cases were drawn only from lots which would later be used for processing.

Description of samples. The fruit for each sample was chosen at random from several bushel apple containers being held in storage. Between 20 and 25 fruit comprised a sample. All samples taken were ungraded fruit of good processing quality and condition. Color bud sports, some of which were used in this experiment, were included with their respective varieties. After samples had been drawn, they were transported to Blacksburg and placed immediately in cold storage at 0° to 3.3° C. (32° to 38° F.). Maturity of samples used varied widely. However, it is believed that maturity and condition of the samples were, in general, representative of the fruit which is received at processing plants in Virginia.

Preparation of samples. Samples were prepared within a week after their arrival in Blacksburg. Approximately 10 apples from each sample were picked out and tested for firmness. For this test a Magness-Taylor pressure tester equipped with a plunger 7/16-inch in diameter was used. A section of peel about ¼-inch in diameter was sliced off each of these apples from the unblushed portion of the fruit. The firmness of the flesh, expressed as resistance against applied pressure and measured in pounds, was recorded. An average was taken for the lot.

The punched apples were then peeled, cored, and sliced. A mechanical hand apple peeler was used. Three slices were taken from each apple and combined, making a composite tissue sample of about 30 slices. This sample was then frozen in hermetically closed Mason jars and kept at about − 23° C. (− 10° F.), until its ash and pectin contents could be determined.

The remainder of the prepared apples were juiced using a Sweden Speed Juicer. The centrifugal action of this juicer produced a juice yield varying from 50 to 60%. Next, the juice was strained through 8 layers of previously washed and dried cheesecloth. Part of the juice was set aside in tightly closed glass containers and stored at − 23° C. (− 10° F.) until its sugar content could be determined. In the fresh juice that was left, pH, total acidity, specific gravity, and soluble solids were determined immediately.

Analytical methods. Determinations were done in duplicate. Ash and pectin were determined in apple flesh. Apple juice was used for all of the other determinations.

(a) pH. pH values were obtained using a Leeds and Northrup model number 7662-A1 Portable pH Indicator Assembly.

(b) Total Acidity. Total acidity was determined in duplicate by titration with N/10 sodium hydroxide solution as indicated in Methods of Analysis of the Association of Official Agricultural Chemists (5). Results were expressed as percent malic acid.

(c) Soluble Solids. Soluble solids content was determined using an Abbe table refractometer. The refractive index was corrected for temperature and converted to percent sucrose from tables.

(d) Sugars. Reducing sugars, sucrose, and total sugars content were found by the Quisumbing-Thomas method for determining invert sugar *(5)*, and cuprous oxide content was found by the volumetric permanganate method *(5)*. First, the reducing sugars content was determined by the above methods, then total sugars content was determined in the same manner as for reducing sugars after hydrolysis with hydrochloric acid. Sucrose was calculated by difference.

(e) Ash. The method used for determining ash content was that given in Methods of Analysis of the Association of Official Agricultural Chemists *(5)* except that a 15-g. sample was used instead of a 25-g. sample.

(f) Specific Gravity of the Juice. A Westphall balance was used. The temperature of the samples was adjusted to 20° C. (68° F.) ± 1° C. immediately before the determination.

(g) Pectin. The calcium pectate method of Carre-Haynes *(11)* was used, as modified by Hinton *(23)*. For extracting pectin from apple tissue, the method given in A.O.A.C.

(5) for extracting samples for pectic acid determination was followed.

(h) Tannin. The method followed was that of the A.O.A.C. *(5)*, as modified by Hartman *(22)*.

RESULTS

Table 1 shows a statistical analysis of the results obtained. In Figures 1, 2, 3, and 4, changes in ash, pH, soluble solids and total acidity with decreasing firmness are comparatively shown for the varieties Golden Delicious, Rome Beauty, Stayman, and York

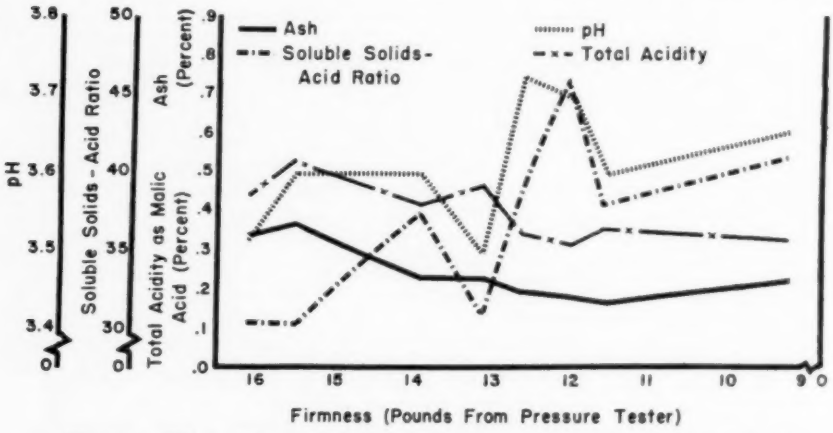

Figure 1. Effect of firmness of Golden Delicious apples upon ash content, pH value, soluble solids-acid ratio, and total acidity.

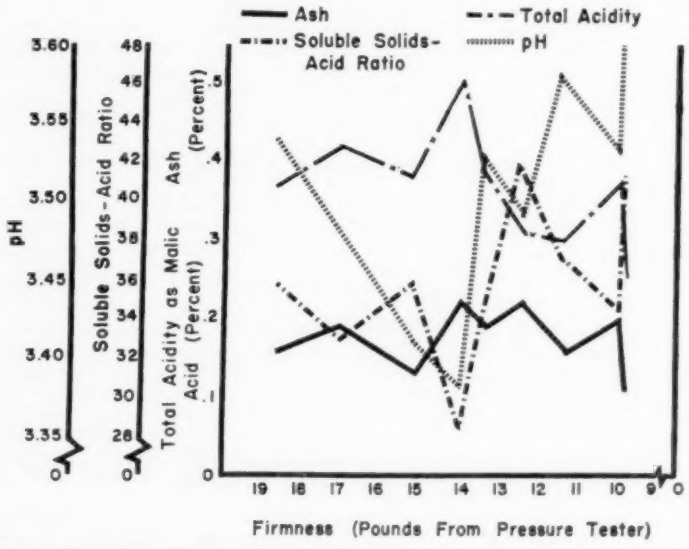

Figure 2. Effect of firmness of Rome Beauty apples upon ash content, pH value, soluble solids-acid ratio, and total acidity.

TABLE 1

A Statistical Analysis on Values Obtained for Several Chemical Determinations and Firmness Made on Ten Varieties of Virginia Processing and Table Apples

	Firmness	pH	Acidity as malic	Soluble solids	Soluble solids-acid ratio	Reducing sugars	Sucrose	Total sugars	Ash	Sp. Gr. of juice	Tannin	Pectin as Ca Pectate
	(Lbs.)	*(Val.)*	*(%)*	*(%)*	*(Ratio)*	*(%)*	*(%)*	*(%)*	*(%)*		*(%)*	*(%)*
Alb. Pippin (6) [2]												
$\bar{x}$ [3]	16.5	3.36	0.71	15.61	22.12	7.90	4.50	12.40	0.25	1.0600	0.03	0.57
s [4]	2.1	0.08	0.08	2.28	2.59	1.13	1.16	2.03	0.03	0.01	0.01	0.06
Delicious (14)												
$\bar{x}$	13.0	3.99	0.25	15.46	62.45	9.59	3.58	13.17	0.26	1.0641	0.03	0.41
s	1.4	0.10	0.04	1.83	11.40	1.39	0.59	1.53	0.08	0.01	0.01	0.06
Golden Del. (9)												
$\bar{x}$	12.9	3.62	0.40	14.60	37.19	8.43	3.66	12.08	0.25	1.0565	0.03	0.64
s	2.0	0.08	0.08	1.32	5.37	0.65	0.60	0.89	0.07	0.00 [5]	0.00 [5]	0.15
Grimes Golden (5)												
$\bar{x}$	11.6	3.50	0.50	15.26	31.13	6.97	4.57	11.54	0.23	1.0557	0.04	0.62
s	1.8	0.05	0.08	1.08	5.16	1.20	1.65	1.28	0.03	0.00 [5]	0.00 [5]	0.05
Rome Beauty (10)												
$\bar{x}$	13.4	3.50	0.36	12.87	36.46	7.16	3.49	10.65	0.18	1.0455	0.04	0.56
s	2.9	0.07	0.07	1.27	4.63	0.77	0.55	0.80	0.06	0.00 [5]	0.01	0.11
Stayman (11)												
$\bar{x}$	12.7	3.40	0.60	14.46	24.33	7.11	4.87	11.98	0.28	1.0558	0.03	0.61
s	1.7	0.04	0.08	1.99	3.35	1.15	1.48	2.03	0.09	0.01	0.00 [5]	0.14
Winesap (11)												
$\bar{x}$	15.1	3.44	0.59	14.49	24.66	8.37	3.44	11.81	0.28	1.0549	0.04	0.76
s	1.5	0.08	0.07	1.80	3.33	0.93	0.83	1.08	0.04	0.01	0.01	0.07
York Imperial (9)												
$\bar{x}$	20.8	3.39	0.72	15.23	23.02	8.07	4.31	12.38	0.24	1.0566	0.03	0.53
s	3.6	0.13	0.25	2.45	7.42	1.04	1.53	1.92	0.06	0.01	0.01	0.05
Jonathan (3)												
$\bar{x}$	9.4	3.48	0.53	13.71	26.03	8.68	2.23	10.91	0.23	1.0528	0.03	0.57
s	0.38	0.03	0.05	1.43	0.55	1.06	0.78	1.78	0.02	0.81	0.03	0.06
Lowry (3)												
$\bar{x}$	13.0	4.25	0.22	17.46	78.84	9.64	3.59	13.23	0.25	1.0647	0.03	0.32
s	0.49	0.09	0.02	0.80	4.54	0.03	0.64	0.83	0.03	0.01	0.01	0.03

[1] Indicates resistance of tissue against applied pressure.
[2] Number of samples per variety.
[3] Mean value.
[4] Standard deviation.
[5] Value smaller than 0.005.

Imperial. Figures 5, 6, 7, and 8 compare changes in soluble solids. Figure 9 shows the relationship between total acidity and firmness found in four of the more important varieties of Virginia processing apples.

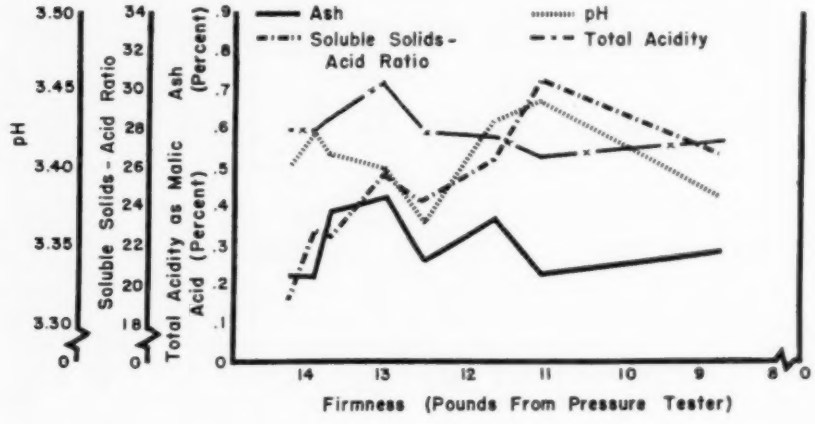

Figure 3. Effect of firmness of Stayman apples upon ash content, pH value, soluble solids-acid ratio, and total acidity.

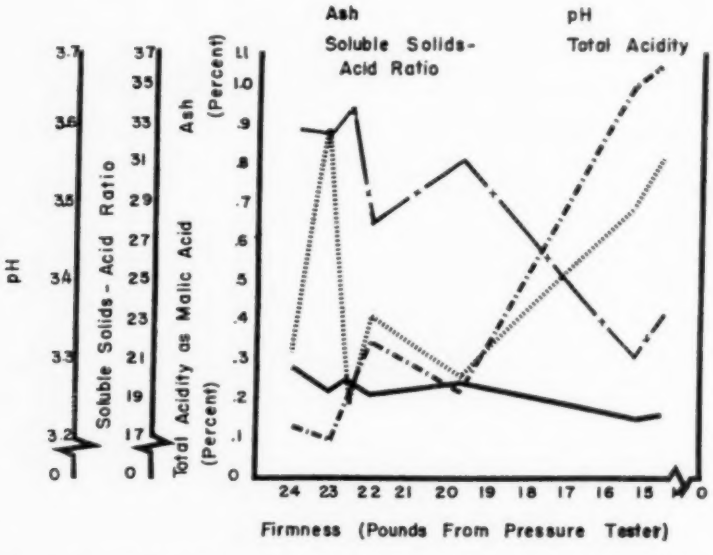

Figure 4. Effect of firmness of York Imperial apples upon ash content, pH value, soluble solids-acid ratio, and total acidity.

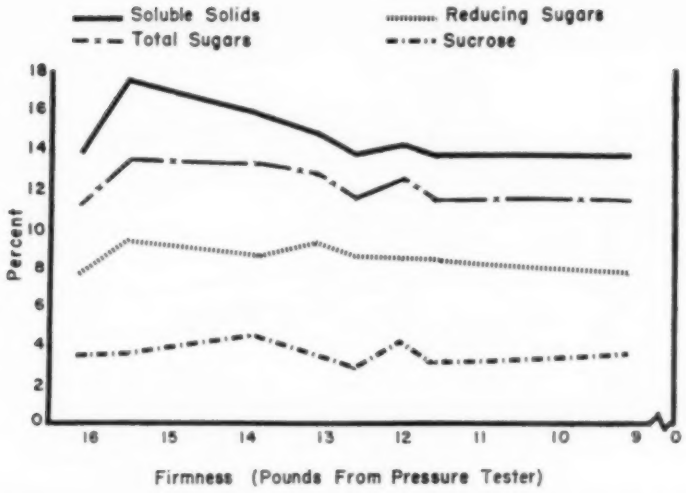

Figure 5. Effect of firmness of Golden Delicious apples upon soluble solids, total sugars, reducing sugars, and sucrose content.

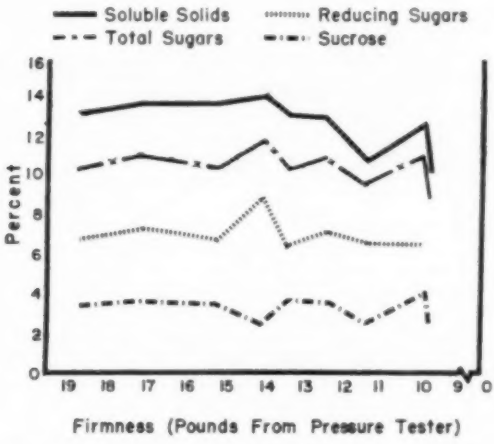

Figure 6. Effect of firmness of Rome Beauty apples upon soluble solids, total sugars, reducing sugars and sucrose content.

Hydrogen-ion concentration. pH values showed considerable variations between varieties, but, for the most part, the differences within varieties were small. Varietal difference was especially evident between the varieties Lowry (pH average of 4.24) and Delicious (pH average of 3.99) and the varieties Albemarle Pippin, Stayman, and York Imperial, the three of which ranged from 3.37 to 3.40. The range in pH within varieties was greatest in York Imperial apples, which for one standard deviation about the mean pH showed 0.13. For Grimes Golden and Stayman the limits were very narrow within

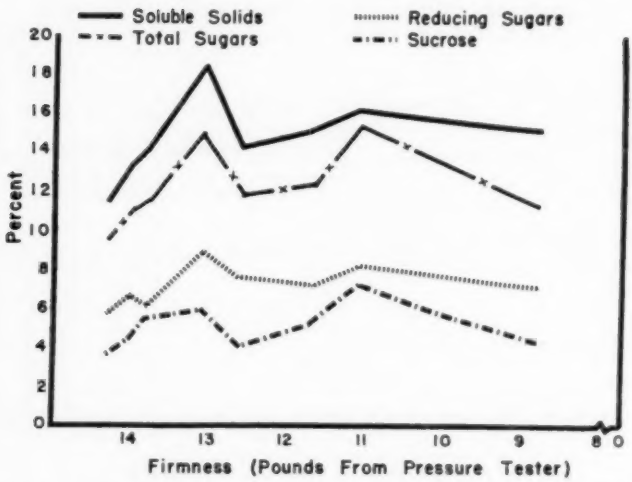

Figure 7. Effect of firmness of Stayman apples upon soluble solids, total sugars, and sucrose content.

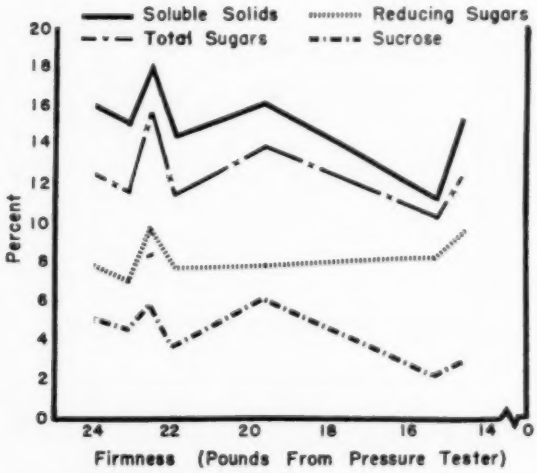

Figure 8. Effect of firmness of York Imperial apples upon soluble solids, total sugars, reducing sugars, and sucrose content.

the variety, one standard deviation about the mean being 0.05. A negative correlation significant at the 1% level of the average of the means for pH and total acidity was observed from Figures 1, 2, 3, and 4. The calculated coefficient of correlation was — 0.86.

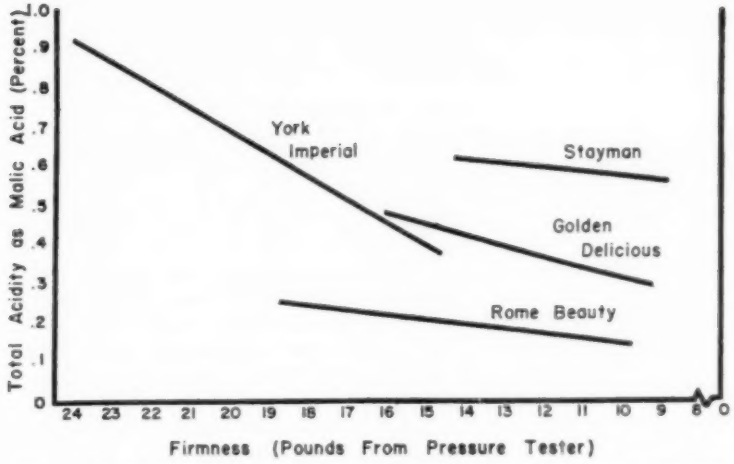

Figure 9. Relationship between firmness and total acidity in four varieties of processing apples.

Total acidity. Total titratable acidity expressed as percent malic acid content of the juice, in general, showed a large variation between varieties, but not within varieties except for York Imperial. Delicious and Lowry were very low in acid content, while Stayman, York Imperial, and Albemarle Pippin were high. York Imperial samples, with a range of 0.318 to 1.074% acid as malic acid and a standard deviation about the mean total acidity value of 0.25%, showed the greatest spread within a variety. It is significant that this same variety exhibited the greatest variation in firmness.

Soluble solids. Soluble solids as determined in the juice of the different varieties of apples showed considerably more variation within varieties than between varieties. For 8 varieties of apples, the average non-sugar soluble solids content (soluble solids content less total sugars content) was 2.73%.

Sugar content. In the apples studied, differences were evident in the sucrose, reducing, and total sugars content of each variety. Total sugars were found to be highest in Delicious and Lowry and lowest in Rome Beauty. Sucrose content averaged highest in Stayman and lowest in Winesap. For reducing sugars content, Delicious and Lowry were high while Grimes Golden, Stayman, and Rome Beauty were low. There was more variation in reducing sugars than in sucrose among varieties. Reducing sugars, sucrose, and total sugars content of the analyzed apples often varied as much within the varieties as between varieties.

Ash content. The varieties showing the highest average ash content, Stayman and Winesap, were 0.10% higher than Rome Beauty which averaged 0.18%. Rome Beauty was much lower in ash content than the average for the varieties studied. Ash varied from a low of 0.12% in a Rome Beauty sample to a high of 0.43% in a Stayman apple sample. Generally speaking, there existed as much variation in ash content within varieties as between varieties. There was a positive correlation between reducing sugars or total sugars and ash content. A correlation coefficient was determined for total sugars and ash content of Winesap apples ($r = +0.625$) and was found to be significant at the 5% level.

Pectin. The highest value was found in the Winesap variety with an average of 0.76%, and the lowest in Lowry apples with an average of 0.32%. More variation was found between varieties than within varieties.

Tannin. Relatively small differences were found in the tannin content of the 10 varieties. The average of the means was 0.033%.

Specific gravity of the juice. The coefficient of correlation between soluble solids and specific gravity of the juice for the average of the 10 varieties was determined and found to be 0.90. It can be assumed, therefore, that changes in specific gravity were determined to the extent of 81% by changes in soluble solids content.

DISCUSSION

The desirable characteristics of processing apples are good typical flavor, firm texture that does not become mushy with cooking, relatively high tartness, acceptable color, and high percentage yield from a given weight of raw applies. Other factors of importance that should be considered either separate from or allied with the 5 factors listed above include: pH and total acidity, soluble solids, sucrose and reducing sugars, astringents, aromatic qualities, pectin, juiciness, texture of flesh, size and shape of the fruit.

Considering the results of all the determinations made on peeled and cored apples in the present study, and results obtained on similar analyses done by other workers on whole fruit, little or no significant chemical composition differences appear to exist between peeled and cored apples and whole apples. Jones and Colver *(24)* helped to substantiate this observation when they stated that the percentage of sugars in the waste, which include stems, cores, seeds, and parings, approximates very closely that contained in the whole fruit.

The composition of the apple varieties studied varied both within and among varieties with firmness. This is an agreement with what many workers have found *(6, 9, 16, 20, 31, 32, 36)*.

Haller *(20)* reports that pressure test determinations are a fairly accurate guide to the ripeness of apples. This same worker, however, states that other factors, such as temperature of the fruit, turgidity and moisture content of the fruit, soil fertilization, soil moisture, thinning, and rootstocks may influence the firmness readings. It is known that apples of the same ripeness for different years exhibit different firmness values *(36)*. Also, composition is not constant for different samples of apples of the same variety due to many other factors.

There was a great deal of difference in firmness values obtained for most of the samples within a certain variety. Most of the samples used, however, ranged from firm to firm ripe. Most of the York Imperial samples ranged from very hard to firm. Within some varieties, samples having nearly identical firmness values possessed a different composition and vice versa.

In general, it was observed that as firmness of apples of one variety decreased (i.e., fruit becomes riper) acidity decreased proportionately, whereas pH increased in the same manner. This is in agreement with results shown by several investigators *(9, 31, 36)*. Soluble solids, total sugars, and reducing sugars decreased with decreasing firmness; sucrose remained relatively constant in the apple varieties studied. It can be assumed that the apple has attained its maximum sugar content when it is harvested in the firm ripe stage of maturity. Afterwards, sugar level decreases. Of course, if the fruit is harvested in the immature state the starch will not have hydrolyzed and the other sugars will not have had the opportunity to reach their maximum level. Even for some of the York Imperial samples, which were very hard and perhaps to be considered immature, the sugar content ran high. The acidity, too, was high in these same hard apples. This would indicate that York Im-

perial apples for processing should be in at least the firm to firm ripe stage of maturity for obtaining the best quality product. The Rome Beauty apples were found to be deficient both in sugar and in acid content. These are two reasons why Rome Beauty apples are not used extensively for canned slices.

In 1955, natural thinning of the trees in many growing areas was brought about by an untimely spring frost. Thinning may have allowed for a somewhat greater build-up of sugars in the fruit. It is quite possible that some of the differences noted in the composition of samples within a single variety were due, at least in part, to the varying growth conditions experienced by each single sample.

Soluble solids-acid ratios have long been used in helping to evaluate apple quality. However, the soluble solids-acid ratios are of a relative nature only. That is, there is no steadfast rule for determining quality directly from soluble solids-acid ratios. Some apple varieties with the same sugar-acid ratios do not have the same eating quality. As Shaw *(31)* stated, Rome Beauty has nearly the same sugar-acid ratio as McIntosh which is much higher in fresh apple eating quality. It is true, however, that each variety possesses a general range for the sugar-acid ratios. For example, Delicious has invariably a high ratio when this variety is mature as compared with most other varieties. St. John and Morris *(30)* maintain that taste of fresh apples as judged by different individuals is a more reliable index of quality than any chemical measures.

Few ash analyses of the apple varieties studied could be found in the literature. For several varieties, no information was available at all. The percent ash in Winesap was reported by several workers: Richards *(29)*, 0.279% ash in whole fruit; Alwood and Davidson *(3)*, 0.31% in whole fruit; and Fellers *(17)*, 0.32% in juice of the whole fruit. The average percent ash obtained from 11 samples of Winesap in the present study was 0.28%. It might be concluded from these data that the percentage of ash present in a given apple variety remains rather constant for that variety and seemingly regardless of the condition of the apples, i.e., as juice, whole fruit, or peeled and cored fruit.

For the Grimes Golden variety, Alwood and Davidson *(3)* reported 0.25% ash in the whole fruit and for the present study, 0.23% ash was found for the average of 5 samples of peeled and cored apples. Alwood and Davidson *(3)* reported 0.25% ash present in the whole fruit of Albemarle Pippin. An identical amount of ash was found in the present study in an average of 6 peeled and cored Albemarle Pippin samples. No previous data have been reported on ash content for the rest of the varieties.

Haller *(21)* found 0.84% total pectic substances in fresh apples. Widdowson *(36)* reported 0.52% total pectic substances expressed as calcium pectate in fresh apples. Macara *(26)* analyzed 28 varieties of apples and found an average 0.75% pectin expressed as calcium pectate, with a maximum value of 1.31% and a minimum of 0.49%. Charley *(14)* reported values ranging from 0.0224 to 0.1780% for pectin expressed as calcium pectate in fresh juice of Bramley's Seedlings apples. In analysis of 8 varieties of Massachusetts grown apples, Fellers *(17)* found values ranging from 0.27 to 0.62% pectin expressed as pectic acid. Lampitt and Hughes *(25)* found an average 1.29% pectin expressed as pectic acid in cooking apples and 0.82% in eating

apples. Carré *(12)* reports values ranging from 0.89 to 1.1% for total pectic constituents determined as calcium pectate. The pectin determinations reported here in general agree well with those reported by the authors mentioned above.

Shaw *(31)* gives equal importance to the amounts of astringents present in the apple to that of sugars and acid. Wright and Whiteman *(36)* reported that total astringents, in general, were highest in Stayman and Winesap, slightly lower in Grimes Golden and immature Yellow Newton and lowest in York Imperial apples. Caldwell *(8)* reports analysis of 21 varieties of French apples grown in two locations in Virginia. He found that tannin content ranged from 0.0912% to 0.5880%, and total astringency values from 0.0912% to 0.8050%. Caldwell also reports in another paper *(9)*, on the chemical composition of the juices of 98 varieties of apples grown in Virginia. The values reported for tannin range from 0.0040 to 0.3290%, and for total astringency from 0.0546 to 0.6400. Values of 0.0377 and 0.0322% for tannin in juices of Virginia grown Delicious apples, and 0.0050, 0.0315, 0.0136% tannin in juices of Virginia grown York Imperial apples are reported by Caldwell *(10)* in a third paper. Neubert *(27)* reports 0.038% tannin found in the juice of Delicious apples. Clague and Fellers *(15)* analyzed the composition of 9 varieties of Massachusetts apples during 2 seasons. They found values ranging from 0.04 to 0.25% tannin. Charley *(13)* reports that the tannin content of ciders is usually 0.12 to 0.14%. The values found in the course of the work reported here agree well with those given in Caldwell's paper.

Figure 9 shows the relationship of firmness and total acidity for the 4 main Virginia processing apple varieties. The regression lines were calculated by equations which were derived from data obtained in the course of the work reported here. As can be seen from Figure 9, the ranges of firmness of the 4 varieties varied, especially that of the York Imperial which differed significantly from the other three. All of the regression lines sloped in the same direction. The slope was similar for Golden Delicious, Rome Beauty and Stayman, all slopes decreasing slightly in acidity as firmness decreased.

SUMMARY AND CONCLUSIONS

The content of several chemical constituents, and other physical and chemical characteristics were determined in 10 varieties of peeled and cored Virginia processing and table varieties of apples grown in several locations throughout the state. The following determinations were made: Firmness of the apple samples, specific gravity of the juice, total acidity, pH, soluble solids, soluble solids-acid ratio, reducing sugars, sucrose, total sugars, pectin, tannin, and ash.

For the chemical determinations made, significant differences were found both within and among varieties. Often the differences in composition were as variable between the samples of one variety as samples of one variety compared with samples of other varieties.

A comparison of firmness values for samples within their respective varieties was made with the results of the different chemical determinations. Total acidity was found to decrease with a loss in firmness, whereas pH and soluble solids-acid ratio increased. In general, soluble solids, total sugar,

and reducing sugars decreased slightly with a decrease in firmness. Firmness values are often not good indicators of the composition of apples. The quality of the apples for processing can be better determined by using data on firmness together with soluble solids. If, in addition, acidity and astringency values are known, a still better prediction of quality can be made.

The York Imperial apple, which is the most important variety to apple processors in the Appalachian area, as compared with other varieties rated, in general, as follows: Very firm, hard apples, high acidity, low pH, average soluble solids, low soluble solids-acid ratio, average sugars, average ash, average specific gravity of the juice, average tannin, and average pectin content.

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CAROTENOIDS IN PASSION FRUIT JUICE
(*Passiflora edulis*, Sims.)

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(Manuscript received March 19, 1958)

There are over 300 species of the genus *Passiflora*, of which only a few have a value as food plants, *viz.*, Purple Passion fruit (*Passiflora edulis*, Sims.), Yellow Passion Fruit *(P. edulis* var. *flavicarpa)*, sweet granadilla or water-lemon *(P. ligularis)*, giant granadilla *(P. quadrangularis)*, bell-apple *(P. lauriflolia)*, West Indian sweet calabash *(P. maliformis)*, red fruited passion vine *(P. foetida)*, green fruited passion vine *(P. ciliata)*, white flowered passion vine*(P. albia)*, and inkberry *(P. suberosa)*. The last four have little, if any, value for human consumption, but are eaten and disseminated by birds. Most of the species not mentioned here are only flowering or ornamental type. *P. edulis*, Sims., the purple skinned fruit, containing an exotic yellowish orange juice embedded in which are numerous small black seeds, is considered to be the most important and is already being commercially exploited in the manufacture of beverages, tropical salads, etc., in Australia, Kenya, South Africa, Hawaii, India, and other tropical countries, but little published information appears to be available on the carotenoid pigment make-up in the juices from any of the aforesaid species, though the subject of plant carotenoids, in general, has been reviewed and discussed from time to time by several workers *(3, 4, 6, 8, 9, 12, 18–21)*. The available information pertains only to the carotene content in *P. ligularis (2, 10)* reported as 0.0 to 0.25 mg./100 g., in *P. quadrangularis (10)* given as 0.004 to 0.019 mg./100 g. and in *P. lauriflolia (7)* as 0.0 mg./100 g. Karrer *et al. (5)* reported the occurrence of lycopene in the red seeds of *P. Coerulea*. Likewise the carotene in *P. edulis* has been reported as 200 I. U. Vitamin A *(13)*, 90 I. U. *(1)* and by us as 901 to 1547 I. U. Vitamin A. *(14–17)*. Thus, despite its being rich in carotenoids, little published information was available on the carotenoid pigment make-up in the juice from *P. edulis*. This paper covers some information on the subject.

Qualitative tests for the water-soluble pigments in passion fruit juice revealed the absence of anthocyanins, and the presence of only traces of water-soluble yellow pigments (flavones). Since these pigments occurred only in very small quantities, no further detailed study was made on them. The present study, therefore, pertains only to the isolation and identification of major carotenoid pigments occurring in passion fruit juice. This was done by separation and isolation of the major carotenoid pigments by column chromatography *(18, 19, 21)* and their characterization by spectrographic data, mixed chromatograms and colour reactions, etc. Preliminary to this study, information was also obtained on the partition of the carotenoids into major groups by the phase-separation technique described by Strain *(19)* and Zechmeister and Cholnoky *(21)*.

EXPERIMENTAL METHODS AND RESULTS

Extraction of juice. Healthy ripe purple plump fruits were washed, halved and the pulp scooped out with the help of stainless steel scooping knives or spoons. The pulp was deseeded by passing the pulp through a motorised pulper fitted with 1 mm. mesh stainless steel sieves. The juice was deaerated and suitable aliquots were withdrawn for experiments; the rest was frozen.

Phase separation method. General methods of extraction, partition, and estimation of carotenoids were essentially those of Strain *(18, 19)* and Zechmeister and Cholnoky *(21)* with slight modifications. Aliquots of 20 ml. of the juice were employed for these studies. The entire pigment portion was taken up in acetone, from which it was transferred to petroleum ether (B. P. 65–70° C.). The petroleum ether extract was washed free of acetone, dried over anhydrous sodium sulphate and made up to a volume of 50 ml.

Total carotenoids. Total carotenoids were estimated by making a further suitable dilution (1:10) with petroleum ether and measuring their absorption in the range 3200 to 6000°A. with a Beckman spectrophotometer (tungsten-filament lamp). This gave an over-all absorption curve of the pigment complex of the juice. Figure 1 represents a typical over-all absorption curve as obtained in a Hardy's Recording Spectrophotometer. Readings at 4250° A. (maxima) gave an idea of the total amount of carotenoids (A) present in the juice.

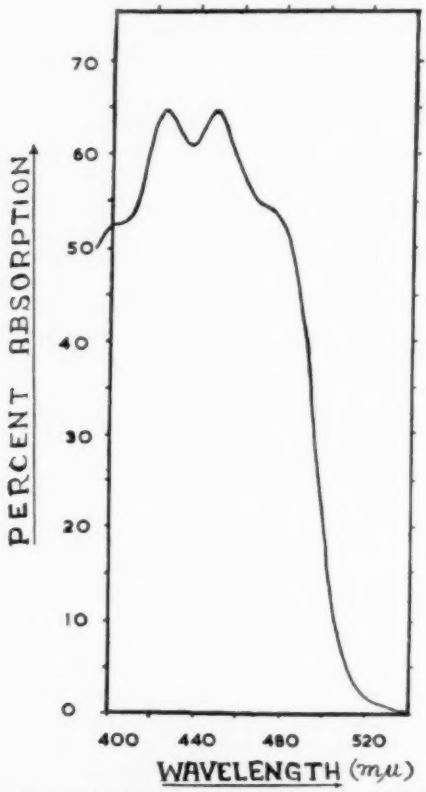

Figure 1. Overall carotenoid pigment picture (absorption spectra) of purple passion fruit juice in petroleum ether (65–70° C.) (absorption curve of petroleum ether extract from 20 ml. of P. F. juice, 1:2 dilution).

Free xanthophylls. These were determined by extracting the original petroleum ether extract (A) containing all the carotenoids with 92% methanol till no more colouring matter was extracted. Methanol extracted the free xanthophylls while xanthophyll esters and carotenes remained in the epiphasic petroleum ether layer, which was then separated, washed with water, dried, made to the original volume taken and read at 4250° A. (B). The difference between the first and the second determination gave xanthophylls (A-B).

Xanthophyll esters. Pigments left in petroleum ether in (B) were saponified with 5 ml. of saturated methanolic potash and held in refrigerator overnight. Next day, the alkali was washed off with water and the petroleum ether was extracted as in (B) with 92% methanol in order to extract the xanthophylls resulting from the saponification of their esters.

Epiphasic non-saponifiable pigments. The petroleum ether (C) obtained from the above was washed with water, dried and treated as in A and B. The reading of (C) gave the non-saponifiable fraction. The xanthophyll esters were obtained by difference (B-C).

The experiment was conducted in triplicate. Only the average values are presented in Table 1. However, as a result of a number of trials conducted, considerable range of variation in the content of total carotenoids and the components thereof was noticed. The same are included in Table 1.

TABLE 1

Partition of the carotenoid pigments between methanol (92%) and petroleum ether

Sl. No.	Carotenoid pigments	Optical density [1]	As per cent of total carotenoids	Range of variation in carotenoids
1	Total carotenoids	0.650	.....	0.26 to 0.81[1]
2	Free xanthophylls	0 140	21.54	10.26 to 21.54%
3	Xanthophyll esters	0.205	31.54	11.11 to 34.57%
4	Epiphhasic non-saponifiables	0.305	46.92	45.74 to 76.30%

[1] Results expressed as optical density at 4250° A. of petroleum ether extract from 20 ml. of fresh passion fruit juice (1:25 dilution).

Further, a similar study of the carotenoid make-up of the juice from passion fruits at 3 different stages of maturity was also made. Since the juice from the raw green fruits contained negligible amount of these pigments, it was not included in the study. The mean results of a typical experiment are presented in Table 2.

TABLE 2

Carotenoid pigment make-up of freshly extracted juice from passion fruit at different stages of maturity

	Stages of maturity					
	Pale Green		Half Purple		Purple	
Carotenoids	O.D.[1]	% of total	O.D.	% of total	O.D.	% of total
Total carotenoids	.330	.....	.540	.....	.710	.....
Free Xanthophylls	.045	13.51	.068	12.59	.073	10.26
Xanthophyl esters	0.65	19.72	.098	18.11	.128	18.09
Non-saponifiables	.220	66.77	.374	69.30	.509	71.65

[1] O.D. = Optical density of petroleum ether extract from 20 ml. juice (1:25 dilution) at 4250°A.

Column chromatographic study of carotenoids. The method of preparation of the pigments was essentially the same as described earlier. Pigments from 2000 ml. of the saponified juice were taken up in 20 ml. of petroleum ether. For large scale work, larger volumes up to 500 ml. of juice were employed. The columns were 11.5 cm. long and 2.5 cm. in diameter. The adsorbant used was a mixture of magnesium oxide and supercel. The filtration was slow with mixtures of 1:1 and 1:2. A mixture of 1:3 gave good results.

Twenty milliliters of the petroleum ether extract containing all the carotenoid pigments was used for adsorption. The tube was kept covered with brown paper closed at the top to prevent the phototchemical action during the development. The column was first developed with petroleum ether and the weakly adsorbed carotenoids were washed down and collected separately. For the next few bands, the petroleum ether containing 3% acetone was used as a developer and the development was completed with petroleum ether containing 6% acetone. As many of the different bands as possible were run down by the above developer. The bands strongly adsorbed on the column were then segmented as free as possible from the adjacent bands and eluted with acetone and later transferred to petroleum ether.

The various eluates were made acetone free, dried as usual, and made up to definite volume (20 ml.) with petroleum ether. The absorption of each eluate was measured separately in a Beckman Spectrophotometer from 3200° A. to 6000° A.

Several chromatograms were necessary to separate each of the carotenes from the complex mixture. The relative position, colour, absorption maxima and the possible names of the carotenoids on the magnesium oxide-super-cel column are given in Table 3.

TABLE 3

The relative position, absorption maxima and colour on a MgO-super-cel column of carotenoid pigments in passion fruit juice

Zones	Colour	Band length (mm.)	Absorption maxima (mμ)	Identity of pigments	Approx. % of each
1	Yellow	10	400,425,450	Unidentified	7.6
2	Yellow	11	400,425,445		8.0
3	Light orange red	10	400,425		9.3
4	Light yellow	8	380,400,425	Z Carotene	8.0
5	Yellow	40	425,450,480	β Carotene	57.8
6	Yellow	15	420,445,475	α Carotene	7.5
7	Colourless	—	345, 365	Phytofluene	—

DISCUSSION

Carotenoid pigment make-up. A study of Table 1 reveals that passion fruit juice contains a complex mixture of pigments in which, unlike the orange juice *(11)*, the non-saponifiables usually dominate and range from 45.74 to 76.30% of the total carotenoids.

It will further be of interest to note from Table 2 that with the development of the external purple colour of the fruit skins, there is a simultaneous gradual building up of the carotenoids, the respective values for the 3 stages of maturity, being 0.33, 0.54 and 0.71 (optical density of 1:25 dilution of petroleum ether extract from 20 ml. of the juice). However, during subsequent studies, considerable variation was noticed in the carotenoid pigment make-up of passion fruit juice extracted from fresh fruits at different stages of maturity. This variation, apart from other agri-cum-horticultural factors, may in part be attributed to the difficulty in obtaining fruits for examination of precisely the same stage of maturity.

ISOLATION AND IDENTIFICATION OF MAJOR CAROTENOIDS

Alpha, beta, zeta carotenes and phytofluene. Pigments 4 to 7 (Table 3) were eluted comparatively easily with petroleum ether containing 3 to 6% acetone. The identity of alpha and beta carotenes was established by their respective spectroscopic pictures and by mixed chromatograms of each of the respective carotenes isolated from carrots. Z-carotene and phytofluene were

likewise identified from their characteristic absorption curves which are well established. These pigments are reported for the first time in passion fruit juice.

Unidentified pigments. The presence of the pigments in small amounts with close absorption affinities have so far prevented their clear cut separation. It seems probable that pigments 1 and 2 at the top of the column might be isomeric forms. The pigments had characteristic peaks at 400, 425, 445 mμ and 400, 425 and 450 mμ respectively. Pigment 3 had the absorption maxima at 400 and 425 mμ. The amount of these individual pigments were too small for further detailed studies.

In conclusion, passion fruit juice, contains a complex mixture of carotenoid pigments in which Beta carotene dominates.

SUMMARY

Passion fruit juice (*Passiflora edulis*, Sims.) has been reported to contain a complex mixture of carotenoid pigments. Employing the phase separation method, the following range of variation was noticed in the major groups of carotenoid pigments: Free xanthophylls 10.3 to 21.5%, xanthophyll esters 11.1 to 34.6% and epiphasic non-saponifiables (mostly carotenes), 45.7 to 76.3% of the total carotenoid pigment make-up.

By employing Tswett column chromatographic technique with suitable modifications and by a study of the absorption spectra of the isolated pigments, their colour reactions and mixed chromatograms, the occurrence of the following pigments have been reported: Phytofluene, alpha-carotene, beta-carotene and zeta-carotene. Three minor pigments having the following maxima, yet remain unidentified (i) 400, 425 mμ (ii) 400, 425, 445 mμ (iii) 400, 425, 450 mμ.

Acknowledgment

Grateful thanks are due to Dr. V. Subrahmanyan, Director, Central Food Technological Research Institute, Mysore, for the keen interest in this investigation, and to Mr. C. P. Natarajan for going through the manuscript.

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FURTHER STUDIES OF THE FACTORS AFFECTING THE EXTRACTION OF COLOR AND TANNIN FROM RED GRAPES

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(Manuscript received April 21, 1958)

In a previous study *(1)* fermentation, sulfur dioxide, alcohol and time were found to be the principal factors (besides temperature) in color and tannin extraction. However, this study did not fully define the effect of each of these, nor did it give sufficient data for use of some of the practical applications suggested by the results obtained. Because of this, further studies were made during 1957.

EXPERIMENTAL

Grapes used in these studies were grown in the University vineyards at Davis and Oakville.

Must treatment. All experiments were made with 5-gallon lots in 20-gallon open earthenware crocks. Sufficient individual 5-gallon lots were prepared to give the desired number of samples. The entire contents of each crock were used as an individual sample to ensure the same juice to pomace ratio for all samples in a given experiment. Uniformity of the lots was ensured by crushing into a 50-gallon drum, stirring the contents, and then ladling, while stirring, one quart amounts of must into each crock in turn until 20 additions had been made.

Unless otherwise noted additions of sulfur dioxide or alcohol were made immediately after the must had been placed in the crock, and each lot was stirred twice daily during the entire course of the experiment. Where fermentation was desired a 1% culture of the Montrachet strain of *Saccharomyces cerevisiae* var. *ellipsoideus* was added 4 hours later. Where fermentation was not desired 40 p.p.m. of Captan (N-trichloromethylmercapto-4-cyclohexene-1, 2-dicarboximide), which was shown in previous work (1) to have no effect on color, was added. All experiments were conducted at comparable temperatures in the range of 18-26° C.

Sampling. One pint samples of the screened 5-gallon lots were removed for analysis.

Analyses. The samples of juice were treated and then analyzed using the procedures reported by Berg and Akiyoshi (1). Since the object of this study was to determine the actual effect of various treatments on color, the color data reported here is that obtained after the addition of hydrogen peroxide to remove the masking effect of sulfur dioxide.

RESULTS AND DISCUSSION

Six experiments were run in this study. Figures 1-4 give the brightness (%Y) of the juices plotted against alcohol or time. In these figures identical symbols at the plotted points in each experiment indicate that the samples were drawn at the same time.

To simplify presentation of the data only brightness (%Y) of the color characteristics is given. It was selected as the best single expression of color density. A decrease in (%Y) indicates an increase in color density. Tannin data are not given as, in general, the tannin content closely followed the trends in color content.

Table 1 gives the must analysis of the various lots of grapes used in the experiments. When more than one number appears in the same line in the

TABLE 1
Must analysis of the lots of grapes used

Experiment No.	Grape variety	° Brix	pH	Total acidity g./100 ml. as tartaric
1, 4	Zinfandel	20.2	3.42	0.85
2, 6	Carignane	21.8	3.59	0.77
3	Cabernet Sauvignon	23.3	3.65	0.58
5	Carignane	21.9	3.52	0.77

column headed "Experiment No." it indicates that these experiments were run on the same lot of grapes.

Effect of fermentation. The object of these experiments was to determine if fermentation factors other than alcohol, temperature, time and sulfur dioxide had an effect on color and tannin release. By keeping all possible factors constant except fermentation its effect can then be measured.

As alcohol was known to be a major factor in color and tannin extraction the writers, in previous experiments *(1)*, had endeavored to keep its effect constant by fortifying one series of samples to various alcoholic contents and then fermenting another series to the same alcoholic contents. Though the fortified series consistently had more color and tannin this finding was questionable because of the time factor involved. The samples of the fortified series received additions of alcohol at the start of the fermentation while all the fermented samples started at 0% alcohol and attained equal alcohol content only at the time of sampling. Thus, even though the contact time for the corresponding samples of both series was the same, the alcoholic contents during that time were different until the moment of sampling.

Experiments 1-3 were designed to minimize variations in the effect of time. Amounts of water equivalent to an addition of 0.1, 3, 6, and 12% alcohol were added respectively to crocks Nos. 1, 2, 3, and 4 in the series to be fermented. With the series to be fortified 0.1% alcohol was added to crock No. 1 and 0.5% to the other three. Captan was added to all lots of this series to prevent fermentation, and 100 p.p.m.of sulfur dioxide added to all lots of both series. Crock No. 1 of both series was screened when the fermentation had produced approximately 0.5% alcohol. Approximately 0.5% alcohol was added to the remaining crocks in the fortified series each time the alcoholic content increased 0.5% in the fermentation series. The No. 2 crock of both series were screened at around 3% alcohol and the No. 3 crocks at approximately 6% alcohol. From this point on alcohol was added in 1% amounts. All crocks of both series were thoroughly stirred each time alcohol was added. With this procedure the alcoholic contents of the fermentation series was higher, until the moment of sampling, than the corresponding lots of the fortified series.

Figure 1 shows that added alcohol generally results in greater color extraction than equal amounts of alcohol produced by fermentation. This offers strong evidence that alcohol is the factor responsible for the extraction of color and tannin during fermentation. However, these data also illustrate that other fermentation factors do have an effect. They either inhibit color and tannin extraction, or cause an appreciable loss in the amounts released. The latter supposition is favored as the color content of the fer-

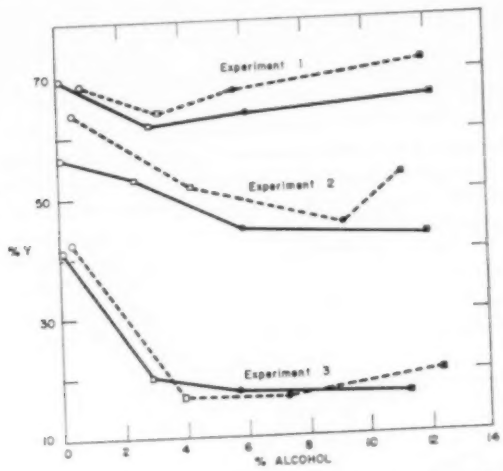

Figure 1. The effect of fermentation. Broken lines — fermented, solid lines — not fermented.

mented juices was less in all 3 experiments. In addition, in all three of the experiments a point of maximum color was reached in the fermenting musts after which the color content decreased; whereas, in the fortified musts the color continued to increase in all but one of the experiments.

Two possible explanations are offered for this effect of fermentation. An increased hydrolysis of the anthocyanins present resulting in their transformation to the less-stable anthocyanidins. Or, the fermentation conditions cause an increased destruction of the pigments with a greater resultant color loss. Both effects, of course, could be operating at the same time.

Differences in behavior noted in the 3 experiments are ascribed to variations between grape varieties. Reference to Table 1 shows that 3 different varieties were used. Though Kepner *et al. (2)* found the same pigments present in the 3 varieties, the relative amounts of the various pigments were not determined. Thus, the noted differences could be due to varying percentage of anthocyanidins in the three varieties. Quantiative determination of the pigments originally present in the grape and at various stages during extraction is needed to explain these differences in behavior.

Effect of sulfur dioxide during fermentation. In previous work by the writers *(1)* the color stability of sulfited (100 p.p.m.) and unsulfited Carignane was found to be equal, with the sulfited must having the greater color content. With Zinfandel musts the one fermented with 250 p.p.m. of sulfur dioxide had the greater initial color, but lost color so rapidly that at the end of the fermentation its color was only slightly greater than that of the must with 100 p.p.m. which lost color at a much slower rate. This raised the question as to whether the difference in color stability was a varietal characteristic, or whether the stability of the color extracted was a function of the concentration of the sulfur dioxide.

Comparison of the fermentations at 0 and 100 p.p.m. of sulfur dioxide (Figure 2) seems to show that the difference in color stability was a varietal characteristic. Further evidence for this is given by Experiments 1 and 2 (Figure 1) where it is seen that the fortified Carignane must was much more stable than the corresponding Zinfandel must.

Comparison of the 3 curves given in Figure 2 also shows that with Zinfandel musts, though more color is extracted with sulfur dioxide, the color stability is inversely proportional to increasing concentrations of sulfur dioxide. It is suggested that this was due to increased hydrolysis of the anthocyanins, and to increased irreversible binding (or destruction) of the pigments. It would be expected that both hydrolysis and irreversible pigment binding (or destruction) would increase not only with increasing sulfur dioxide concentrations, but also with increasing reaction time.

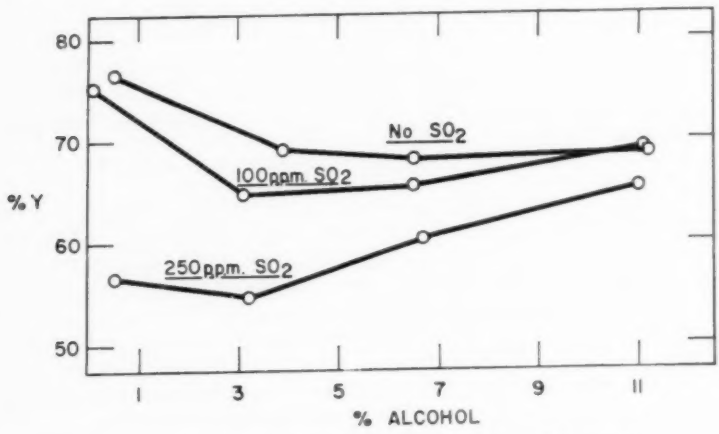

Figure 2. The effect of sulfur dioxide on fermenting musts.

Effect of alcohol and time. Extraction of the pigments from grape skins involves both the loss of the semipermeability of the cells in the skins, and diffusion of the pigments through the cell walls into the surrounding liquid. The percentage of cells losing their semipermeability is dependent upon temperature, agitation, the kind and concentration of substances present which affect the semipermeability of the cells, and the reaction time. The ensuing diffusion of the released pigment into the surrounding juice also requires time.

Indications were obtained in previous work *(1)* to support the belief that the degree of loss of the semipermeability of the cells in the skins governs not only the percentage of the total color released, but also determines the diffusion rate. To further test this belief 4 lots of the same must were stored with 100 p.p.m. of sulfur dioxide at 10, 20, 30, and 40% alcohol. Samples were drawn at 12, 24, 48, 72, 96, and 120 hours. As the samples were drawn from the 10% alcohol must they were fortified to 20% alcohol. Before analysis the samples from the 30% and 40% alcohol musts were vacuum distilled to re-

duce the alcoholic content to the same level as that in the samples from the other two lots of must.

As shown in Figure 3, color release and diffusion rate are both dependent upon the degree of loss of the semipermeability of the cells in the skins. More color was released with increasing alcohol concentration and increasing reaction time. Also, at equal diffusion times the amount of color which diffused through the cell walls into the surrounding juice increased with increasing alcohol concentration.

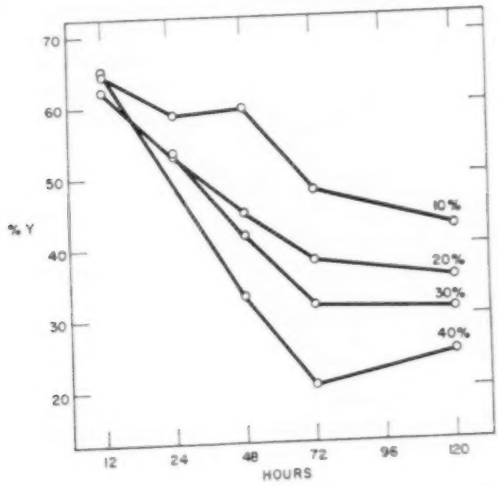

Figure 3. The effect of alcohol and time. The % sign indicates percent of alcohol by volume.

Figure 3 also shows that a minimum reaction time is required at each alcohol concentration to reach equilibrium. Both the increase in rate of color release up to 72 hours and the decrease in rate from that point to 120 hours were a function of the concentration of alcohol. With this particular Carignane must (Experiment 5) color release rate is practically a straight line function up to 72 hours. Evidently a considerable reaction period, even at very high alcohol concentrations, is required to reach equilibrium.

Effect of alcohol and sulfur dioxide. Color extraction during fermentation is a time consuming operation that is costly because of the labor involved and the fermentation tank capacity required. If the color extraction time could be reduced from the present 2 to 3 days, the savings in labor cost and decreased capital investment would be very substantial. Experiment 6 was run to determine the feasibility of increasing fermenter turn-over in red wine production in order to accomplish this.

Two lots of 4 samples each and 2 lots of 1 sample each were prepared from the same must. One lot received an addition of 100 p.p.m. of sulfur dioxide and the second lot 250 p.p.m. Both of these lots were stored for 24 hours at the alcohol concentrations shown in Figure 4. The third and fourth

lots, which served as controls, received additions of 100 and 250 p.p.m. of sulfur dioxide respectively, and then were fermented to approximately 12% alcohol content over a period of 96 hours. All samples of the 4 lots were fortified to 20% alcohol content immediately after drawing.

As shown in Figure 4 the color content of the juices from the non-fermented musts was a function of both the sulfur dioxide and alcohol contents. The color content of the juices from the fermented musts was less than that

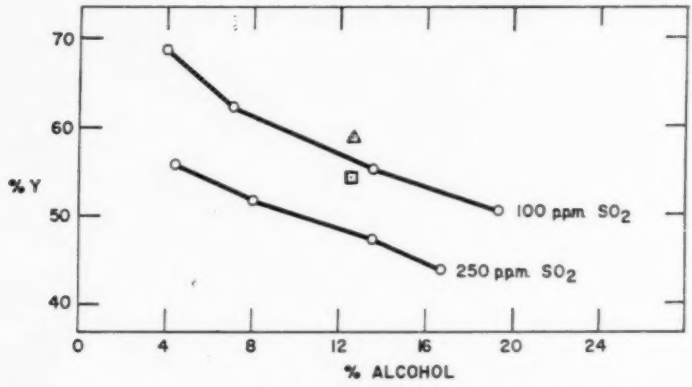

Figure 4. The effect of alcohol and sulfur dioxide. Solid lines — not fermented. △ — fermented to alcohol content shown with 100 p.p.m. of sulfur dioxide. □ — fermented to alcohol content shown with 250 p.p.m. of sulfur dioxide.

of the corresponding non-fermented musts at the same alcohol level. This difference was even more striking in view of the fact that the juices from the non-fermented musts had been diluted approximately 13% more than the juices from the fermented musts by the fortification to 20% alcohol content. In comparison to the 96 hours required for fermentation, equal color was obtained in the non-fermented musts in 24 hours at the 100 and 250 p.p.m. sulfur dioxide levels with respectively 10.6 and 5.4% alcohol contents. Thus, in the production of port wines adequate color extraction can be obtained in 24 hours or less at alcohol contents well below the 20% usually found in this wine type. The writers wish to point out that color is not the only consideration. Before any commercial application of this procedure is considered it is essential that the wines be aged and tasted to determine if they are equal in quality to those produced by present methods.

SUMMARY

In fermentations of non-sulfited musts alcohol is the only factor of major importance in effecting color and tannin release from the grape skins. Alcohol alone produces juices with more tannin and color than does fermentation. In fermentations of sulfited musts maximum color content is obtained prior to the end of the fermentation. Grape varieties vary in their behavior during color and tannin extraction.

With Zinfandel musts the color stability of the juices is an inverse function of the amount of sulfur dioxide added prior to fermentation.

Both color release and diffusion rate are dependent upon the degree of loss of the semipermeability of the cells in the skins. The color and tannin content of the juices from fortified musts increases with increasing alcohol concentration and increasing reaction time. A minimum reaction time is required at each alcohol concentration to reach equilibrium.

More color can be obtained by storing fortified musts for 24 hours than by fermenting the musts to the same alcohol content over a period of 96 hours. The color and tannin content of juices from fortified Carignane musts was directly proportional to both the sulfur dioxide and alcohol concentrations at which they were stored.

A new process has been provisionally suggested for the production of red sweet wines.

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MECHANISM OF COPPER CASSE FORMATION IN WHITE TABLE WINE. III. SOURCE OF THE SULFUR IN THE SEDIMENT.

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(Manuscript received May 3, 1958)

White table wines containing excess of cupric ion may become turbid and subsequently form a sediment containing copper. This occurs under reducing conditions and is accelerated by the presence of free sulfurous acid *(10, 11, 15)* or on exposure to light *(7, 8)*. Trichloracetic acid (TCA) precipitable nitrogenous constituents (presumably polypeptides or proteins) are required *(10, 11, 12, 15)*, but the critical level of these constituents is not known. Copper casse formation was observed in wines containing 15 mg. per liter of TCA-precipitable nitrogen expressed as protein when stored in the dark in presence of added sulfite *(15)*. Kean and Marsh *(11)* observed casse formation in commercial wines containing 300 to 600 mg. of total organic nitrogen per liter of which only 1.5 to 4% was present as protein. They also observed casse formation when wines containing as little as 5 mg. per liter of protein were stored in direct sunlight in absence of oxygen *(11)*. Recently we investigated a commercial white table wine containing 114 mg. of total organic nitrogen per liter of which 0.9 mg. was precipitable with TCA (equivalent to a protein content of 5.6 mg.), but which did not become turbid when exposed to sunlight at room temperature or when stored in the dark at temperatures up to 50° C. (This wine contained 5 p.p.m. of iron, 10 p.p.m. of copper and up to 300 p.p.m. of sulfur dioxide).

The copper complex formed in wines containing sufficiently high concentrations of protein and sulfurous acid dissolves readily when the wine is oxidized by exposure to air or by addition of hydrogen peroxide *(10, 15)*. Wines containing excessively high concentrations of protein when stored in the dark at temperatures above 20° C. or when exposed to direct sunlight for too long a time will form a sediment which does not redissolve. Denaturation of proteins by heat and by light is well established *(8, 16, 18, 19, 20)*. Sulfites also are known to precipitate proteins and to form permanently insoluble precipitates but only at concentrations approaching saturation *(1)*. Complex formation between polypeptides and metal cations such as cupric ion is known to occur and the chemistry of these reactions has been investigated systematically by Dobbie and others *(4, 5, 6)*. Sulfhydryl compounds such as cysteine are known to catalyze polymerization of denatured proteins by increasing the extent to which intermolecular sulfide bonds are formed. This aggregation by the formation of intermolecular disulfide bonds has been observed during heat and urea denaturation of plasma proteins and more recently during ethanol denaturation *(21)*. While the influence of cysteine on precipitation of plasma proteins has been reported to occur in the presence of high concentrations of added ethanol (70% by volume) it is possible that polymerization of this type may occur in wines containing sulfites and cupric salts. Only the turbidity which forms under reducing condi-

tions and which redissolves under oxidizing conditions is considered to be a true copper casse *(10, 11, 12, 15)*.

It has been postulated that reduction of cupric ion to cuprous ion and the reduction of sulfur dioxide to hydrogen sulfide are part of the mechanism of casse formation *(10, 15)*. At the time those investigations were carried out it was not possible to prove unequivocally that reduction of cupric ion occurs, although the presence of cuprous sulfide and cupric sulfide was established by X-ray diffraction data *(15)*. The discovery of a reagent specific for cuprous copper (cuproin) *(9)* makes this possible. Cuproin has already been used to determine the oxidation state of copper in polyphenol oxidase *(14)*, and in various cupric-ion-catalyzed oxidations *(7, 13)*. Investigations of the kinetics of valency change in copper ions during casse formation in wine are in progress. It is also necessary to establish whether the sulfur in the copper sulfide precipitate is derived from reduction of sulfur dioxide or from cleavage of the disulfide bonds in protein. It is well known that in the presence of sulfite, organic disulfides are converted into a thiol compound and a S-sulfonate compound *(2, 3, 8, 17, 19)*. In the sulfite denaturation of proteins containing accessible disulfide bonds such as the keratins *(8, 17)* and insulin *(2)* thiols also form.

This report deals with an investigation in which sodium bisulfite containing the radioactive isotope S^{35} was used to determine whether the sulfur in the copper casse originated from sulfur dioxide or from protein.

EXPERIMENTAL

The white table wine used was a blend of 4 wines prepared at the Enology Laboratory at Davis from 4 varieties of white grapes grown at Davis. These grapes were harvested during the vintage season of 1952 and were crushed and pressed on the day of harvest. The finished wines were obtained early in 1953 and stored at 50° F (10° C) in glass until used. Pertinent data on these wines are given in Table 1. These wines were used in investigations previously reported *(10, 15)*. The blend used in the investigations reported here was prepared during the summer of 1957 by mixing one liter of each of the 4 basic wines and filtering the mixture. The composite wine contained 2.3 p.p.m. of iron, 0.15 p.p.m. of copper and had a total organic nitrogen content of 148 mg. per liter of which the TCA-precipitable nitrogen was 8.91 mg. per liter (equivalent to 55.7 mg. of protein). This was brought to 5.0 p.p.m. of iron by addition of ferrous sulfate and to 12.7 p.p.m. of copper by addition of copper sulfate. The sulfur dioxide content was brought to a total of 450 p.p.m. by addition of sodium bisulfite containing radioactive S^{35}. The labelled bisulfite was added so that the wine contained about 6 microcuries of S^{35} per liter.

The wine was deaerated before addition of sulfite and stored in full 1 liter Pyrex glass stoppered flasks. One lot was maintained at room temperature (about 20° C.) and exposed to direct sunlight; one lot was stored at 20° C. in the dark; one was held at 35° C. with 16 hours of light per day; and one was placed in an oven at 40° C. in the dark. After at least 3 weeks of storage, found previously to be sufficient to produce the maximum turbidity, all 4 flasks were cooled to 5° C. The suspended insoluble matter and that sedimented was separated by centrifuging 40 ml. fractions in a Servall high speed centrifuge at 1600 r.p.m. The combined sediment was resuspended in a centrifuge tube in 40 ml. of cold distilled water and this procedure was repeated 4 times. After the first washing to remove occluded radioactive sulfite the residual activity of a typical dried precipitate was 1.28 counts per second; after the second washing this decreased to 1.09 c.p.s., and on the last 3 washings it was 1.08, 1.07, and 1.08 c.p.s., respectively. Washing with cold distilled water and centrifuging did not cause appreciable losses in weight of sediment or in its composition *(15)*. Each precipitate was then air dried, weighed, and deposited on a 1 cm. diameter aluminum disc.

The beta radioactivity was measured with a Tracerlab Autoscaler with a thin-window

TABLE 1

Source and composition of base wines [1]

Lot No.	Variety	Date picked	Must analysis			
			Condition *	Balling	Total acidity	pH
5652	French Columbard	9/24/52	Good	20.9	1.18	3.35
5678	Chenin blanc	9/26/52	Excellent	19.0	0.94	3.38
5806	Sauvignon blanc	10/7/52	Good	25.1	0.87	3.56
5898	Pinot blanc	10/23/52	Fair	23.1	0.62	3.41

FERMENTATION CONDITIONS

Lot No.	Sulfur dioxide added	Duration, days
5652	76 p.p.m.	9
5678	75 p.p.m.	9
5806	75 p.p.m.	—
5898	75 p.p.m.	17

WINE ANALYSIS

Lot No.	Date	Total acidity	Volatile acidity	pH	Extract	Reducing sugar	Alcohol	Tannin
5652	12/3/52	1.05	0.054	3.40	3.1	0.10	10.5	0.03
5678	12/8/52	0.90	0.045	3.35	2.7	0.05	9.9	0.04
5806	12/31/52	0.72	0.054	3.63	3.0	0.08	12.8	0.02
5898	1/15/53	0.69	0.036	3.31	2.5	0.07	12.3	0.04

[1] These wines were prepared and analyzed in the Enology Laboratory of the University of California at Davis. Professor M. A. Amerine's cooperation in making these wines available to us is appreciated.

Geiger tube. It was assumed that all the activity present was due to the copper sulfide flocculated by wine protein or to strongly adsorbed sulfur dioxide.

The solubility of the copper casse in dilute alkali *(15)* was used to differentiate between radioactive sulfur present as copper sulfides and that which was either strongly adsorbed by the precipitated proteins or formed from the disulfide bonds of the protein after reaction with sulfite. After counting, the dried precipitate was dissolved in an excess of cold 0.1 N sodium hydroxide. The sulfite present was oxidized by the addition of 1 ml. of bromine water and stored for 25 hours at 30° C. The protein and other organic colloids present were precipitated by addition of several drops of 4 N HCl. The acid-precipitated matter was centrifuged, washed, dried, weighed and counted. It still had an appreciable amount of radioactivity due either to adsorbed or chemically reacted sulfite. The clear supernatant solution was light blue in color (owing to the presence of cupric ions) and contained sulfate ions (formed by the oxidation of copper sulfide and adsorbed SO_2). The sulfate present was precipitated as barium sulfate by the addition of 1 ml. of 10% barium chloride and holding at 100° C. for 10 minutes. The barium sulfate was separated out by centrifuging, ignited, weighed and counted. The sulfite present in a 10 ml. aliquot of the wine after removal of the insoluble matter formed was also converted into barium sulfate, ignited, weighed, and counted. The chemical analyses reported for iron, copper, sulfur dioxide, and nitrogen content were made by procedures previously described *(15)*.

RESULTS AND DISCUSSION

Data on the weight of casse obtained under each of the 4 storage conditions and the specific activity of S^{35} are shown in Table 2. This table also summarizes the data on the weight and specific activity of the barium sulfate obtained from the original wine after casse formation, that from the casse, and the activity of the residual proteins obtained by alkali fractionation. The activity of the various preparations is given as total measured activity and also as concentration in terms of counts per minute per milligram of sulfur. If the specific activity of the sulfur of the copper complex is the same as that of the supernatant wine, then the copper complex is formed by interaction between cupric ions and sulfite ions alone and the protein present exercises only a secondary effect. This would be true whether the primary copper complex is cuprous or cupric sulfide or cuprous sulfite. That this is so for casse formed in the light is quite evident from the data presented in Table 2. Both

TABLE 2

Weight, total and specific activity of sediments and barium sulfate preparations

		Weight	Activity c/m	c/m/mg. S
20° Dark	Supernatant wine SO_2	41.5 mg. $BaSO_4$	1212	212
	Casse	14.9 mg. casse	101.5	
	Oxidizable casse S	2.6 mg. $BaSO_4$	51.3	142
	Final ppt'd casse		67.1	
40° Dark	Supernatant wine SO_2	45.0 mg. $BaSO_4$	1322	213
	Casse	29.4 mg. casse	250.5	
	Oxidizable casse S	4.3 mg. $BaSO_4$	91.5	154
	Final ppt'd casse		174.0	
20° Light	Supernatant wine SO_2	43.6 mg. $BaSO_4$	1250	208
	Casse	26.0 mg. casse	302	
	Oxidizable casse S	5.6 mg. $BaSO_4$	146.5	188
	Final ppt'd casse		164.3	
35° Light	Supernatant wine SO_2	43.5 mg. $BaSO_4$	1260	210
	Casse	41.2 mg. casse	466.1	
	Oxidizable casse S	8.5 mg. $BaSO_4$	241.0	204
	Final ppt'd casse		243.8	

at 20° and 35° C. in sunlight the specific activity per mg. of sulfur in the wine supernate and in the bromide-oxidizable copper casse sulfur is the same within the limits of experimental error. In the dark, however, the specific activity of the oxidizable sulfur of the copper casse is appreciably lower than that of the wine supernatant, indicating that reaction of sulfite with proteins leading to the formation of thiols or sulfonates capable of binding copper has occurred. In all cases the acid-precipitable material obtained after alkali treatment contained appreciable radioactivity. Thus, we can calculate the amount of SO_2 adsorbed by the wine proteins. This was greater in the light than in the dark and increased appreciably with temperature.

In the coagulation of proteins by ultra violet light at least 2 steps are involved; a photochemical reaction independent of temperature, followed by flocculation of the denatured protein, the latter reaction having a high temperature coefficient *(16, 18, 20)*. Apparently a similar sequence of reactions occurs in the presence of copper and sulfite ions since the yield of casse obtained in the light and its activity is greater at 35° C. than at 20° C.

The reaction of sulfite with disulfide bonds of proteins has been found to be more complex than that of organic sulfides (cystine). Cecil and Loening *(2)* reported that the reaction of disulfide bonds of insulin with sulfite varies in rate and extent with pH. In the pH range of 2.8 to 5.5 it is slow and only one of the disulfide bonds is split; at higher pH values it is more rapid and more disulfide bonds are split. In the presence of a thiol reactive reagent, however, the reaction is rapid and complete. It is possible that in the reaction of sulfite with wine proteins, copper favors splitting of disulfide bonds. It is not known, however, whether the organic thiols formed would yield copper complexes capable of producing sulfates on oxidation with bromine.

If the wine protein acts simply as a flocculating agent, it should be possible to separate the copper complex from the protein. Lukton and Joslyn *(15)* reported this to be the case for one copper complex which was separable by paper electrophoresis at pH 8 to 9 into copper salt and protein. The radioactive copper protein complex obtained from the composite wine stored at 40° C. in the dark was used in similar electrophoretic experiments. A small quantity of the complex dissolved in 0.1 N sodium hydroxide was spotted on a paper strip in a Spinco Paper Electrophoresis apparatus. After 4 hours at 300 volts and 15 to 20 microamperes in a veronal buffer at pH 9 the paper strip was examined in a Geiger counter to detect any separation of protein from the copper complex. Little if any separation was observed. The small spot remaining at the origin gave a positive test for both copper and protein and had an activity of 44 c./m.; the background count also was 44; the spot which moved to the cathode also was positive for both copper and protein, but had a slightly higher activity, 57 c./m.

In veronal buffer at pH 9 the copper-protein complex was positively charged. Apparently under the conditions used the copper was firmly bound to the protein and the reaction product of sulfite and protein.

The mechanism of the formation of an insoluble copper complex formed in wine in the presence of sulfur dioxide was investigated by the use of sodium bisulfite containing labelled sulfur. The complex formed contained

SUMMARY AND CONCLUSIONS

both sulfur that was oxidizable to sulfate by bromine and sulfur which was not separable by solution in alkali and subsequent acidification. The quantity of the latter formed was greater at 40° C. than at 20° and was greater in the light than in the dark. In the light the copper complex is formed by reduction of sulfite to sulfide with the subsequent formation of insoluble cupric sulfide and its flocculation by protein. In the dark this is true for much of the casse, but some casse is also formed by sulfite denaturation of protein. Such denaturation results in a copper-protein complex capable of yielding sulfate-S on oxidation. The mechanism of this reaction is not known, but it is not due either to a simple sulfite denaturation of protein or to the usual sulfitolysis of protein.

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ANTIBIOTIC RESIDUALS AND MICROBIAL RESISTANCE IN POULTRY TREATED WITH TETRACYCLINES[a, b]

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Many antibiotics have been investigated as possible agents for prolonging storage life of perishable foods; chlortetracycline (CTC) and oxy-tetracycline (OTC) were found acceptable for this purpose. These tetracyclines have received the approval of the United States Food and Drug Administration for use on poultry but with the stipulation that residuals not exceed 7 p.p.m. in the raw tissue and that no residual be present at the time the food is to be consumed.

The tetracyclines retard the growth of the microflora for a time but after several days of storage the number of organisms increases and spoilage results. Subsequent growth of organisms on carcasses treated with tetracyclines could result from the development of an antibiotic resistant population or from a decrease in the activity of antibiotic in tissue to such a level that the organisms were no longer inhibited. An increase in the resistance of organisms growing on chicken processed in the presence of CTC as compared to poultry processed in the absence of CTC has been reported by Ng *et al. (11)*.

The purpose of this work was to determine the residuals of CTC, OTC and tetracycline (TC) remaining in raw poultry tissue after treatment with concentrations of 10 μg./ml. and 30 μg./ml. of the respective antibiotics and also to determine the effect of the residual antibiotics on the resistance of organisms causing spoilage of poultry.

MATERIALS AND METHODS

Thighs and backs of chickens were soaked for 10 or for 120 minutes in aqueous solutions containing 10 or 30 μg./ml. of CTC, OTC or TC. Only thighs were used for determination of residual antibiotics in deep or subsurface tissue. The pieces of chicken were packaged in polyethylene bags and stored at 4° C. In addition, samples of antibiotic solutions used for treating the pieces of chicken were stored at 4° C. and were assayed for any decrease in concentration of antibiotic.

Antibiotic concentrations were determined by microbiological assay using the pad plate method described by Grady and Williams *(7)*. Duplicate determinations of the standard curve and of the unknown samples were made on each plate. Comparison of the average zone diameters of the unknown samples with those of the standard curve indicated the quantity of residual antibiotic.

For the assay, 150 ml. of brain heart infusion agar inoculated with spores of *Bacillus cereus* var. *mycoides* (ATCC 9634) was poured into a pyrex utility dish (13½ x 8¾ x 1¾ in.). The spore suspension generally was of such concentration that 1 ml. of spore suspension per 100 ml. agar gave satisfactory results. After the agar solidified and the

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assay disks were placed on the plate, the plate was refrigerated 1 hour before it was incubated at 30° C. for 18-24 hours. For some of the assays 18-hour broth cultures of *B. cereus* were used as inocula and results similar to those with spores were obtained. However, use of a spore suspension eliminated the necessity of daily transfers and variations which could result from differences occurring in the broth cultures from day to day. The spore suspension was stored at 4° C.

Skin and lean surfaces were sampled by placing ½ in. diameter assay pads[6] on the surface until the pads became saturated with free moisture. Previous determinations had shown that pads would absorb 100-110 mg. moisture when dipped into a solution or the equivalent of 0.1 ml. of solution. When used on the surface of chicken, pads generally absorbed 85-100 mg. of surface fluids if skin or lean flesh surfaces were moist. If surfaces tended to be dry, it was necessary to move the pad to areas adjacent to the initial site of testing to obtain satisfactory moisture content in the pad. Lean surfaces sampled were exposed by removal of skin. It is recognized that the method of sampling surfaces was only a crude quantitative approximation, but it did show the relative decrease of residual antibiotics in the surface fluids and also eliminated the problem of trying to cut out surface samples to a standard depth.

To determine concentration of antibiotic in subsurface tissue, surface tissue was removed with the aid of a scalpel and approximately a 1 g. sample of tissue about ¼ in. below the surface was obtained. Sample was weighed and water added at a ratio of one part tissue to three parts water. The mixture was ground with a mortar and pestle in the presence of 1½-2 g. sand. After the debris had settled, assay pads were saturated with the supernatant liquid and placed on the assay plate.

Samplings were made of skin and lean surfaces and of tissue immediately after treatment with antibiotic solutions, after 6 hours and after 1, 2, 3, 6, 8, 10 and 14 days. Samples of antibiotic solutions used for dipping were stored also at 4° C. and sampled at the same time intervals.

For studies on development of bacterial resistance to the tetracycline antibiotics, halves of eviscerated birds were immersed for 120 minutes in solutions containing 10 μg./ml. of CTC, OTC or TC. Controls were prepared by soaking the corresponding halves in water for 120 minutes. Half carcasses of birds were stored at 4° C. in polyethylene bags. Swab samples of the skin surface were taken at 0, 2, 5, 10 and 12 days. Spread plates were made on nutrient agar and incubated at 15° C. for 72-96 hours. Colonies from spread plates were transferred by the replicate plating technique *(9)* to other nutrient agar plates containing CTC, OTC or TC at levels of 0, 0.5, 1.0, 2.5 or 5.0 μg./ml. These plates were incubated for 36-48 hours at 15° C. It was necessary to select plates that were not overcrowded in order to obtain suitable resolution of colonies; plates containing no more than 100 to 125 colonies were found to be most suitable for this type of transfer.

RESULTS AND DISCUSSION

There was no appreciable decrease in potency of any of the 3 tetracycline solutions used to soak chickens during two weeks of storage at 4° C. The solutions were slightly acid; the pH values ranged from 6.0 to 6.8. CTC has been reported by Lepper *(10)* as being stable in distilled water when stored at 4° C.

Figure 1 shows the levels of CTC, OTC and TC present in the liquid on the skin surface; each point on the curves showing concentration of antibiotic represents an average of 10-12 determinations. During the first 48 hours the concentration of antibiotics on the skin surfaces decreased rapidly; after 2 days no pronounced decrease was observed. At the end of the experimental period, concentrations of TC were highest; those of OTC were intermediate and those of CTC were lowest. The concentrations recovered of the respective antibiotic were dependent on the treatment. The same relationships were observed when comparisons were made of the recoveries from lean tissue

[6] Carl Schleicher and Schuell Company, Keene, New Hampshire.

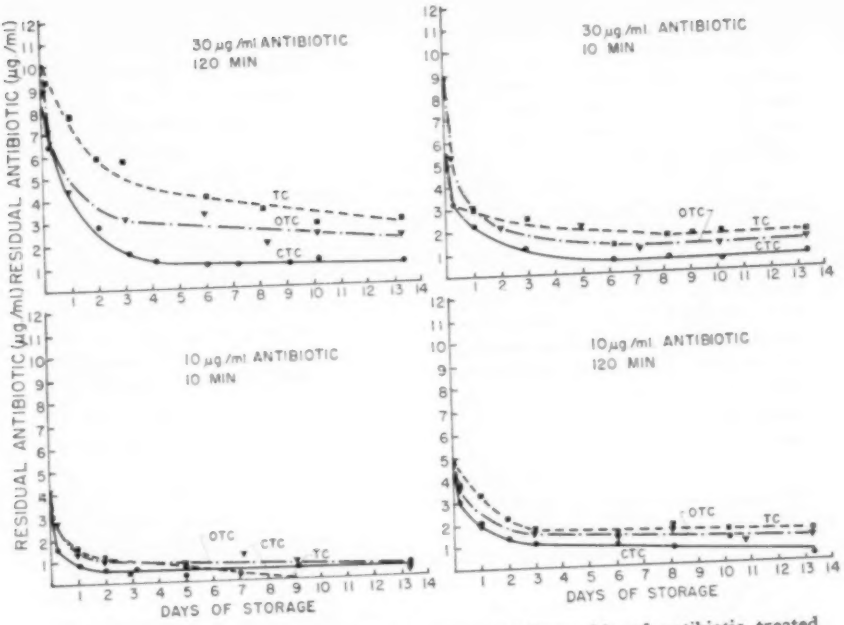

Figure 1. Residuals of tetracyclines recovered from skin of antibiotic treated poultry stored at 4° C.

surfaces and subsurface tissue. Therefore, it appeared that in chicken flesh TC was more stable than OTC, and OTC more stable than CTC. In fact, TC has been shown to be the most stable, OTC intermediate in stability and CTC least stable of the three over a pH range of 2.5 to 9.0 when heated at 100° C. for 15 minutes or when incubated at 37° C. in phosphate buffer *(4, 6)*. Wright et al. *(15)* showed also that during storage at 5° C. in water or saline at a slightly acid pH or in citrated plasma, CTC deteriorated most rapidly and TC least rapidly of the three. Thus, the initial decrease in the concentration of these antibiotics in poultry tissue could be attributed to inactivation of the antibiotics and to diffusion of the antibiotics throughout the flesh.

The curves in Figure 1 also show that the concentration of antibiotic found on the skin varied directly with the concentration of antibiotic solution used and length of treatment time. For example, the residual on the skin was higher when 30 μg./ml. of antibiotic was used than when 10 μg./ml. was used; also the residual was higher after treatment for 120 minutes than after 10 minutes. Recoveries from the lean surfaces are not shown since the concentrations of the residues parallel those for skin surfaces. The recoveries from the lean surfaces during the first 48 hours would average about 1 μg./ml. less than that from the skin. After this time there was no appreciable difference between the two.

Abbey *et. al. (1)*, Windlan *et al. (14)* and Broquist and Kohler *(5)* pre-

sented data indicating that the level of residuals of CTC in poultry tissue varied with the concentration of antibiotic used. Silvestrini *et al. (13)* and Windlan and his co-workers *(15)* demonstrated also that the levels of CTC residues varied from one tissue to another. For instance, the level was frequently highest in skin tissue and frequently lowest in breast tissue. Anderson, *et al. (2)* treated chickens with solutions containing 10 μg./ml. of CTC and after 3 days storage could recover 0.91 and 0.93 μg./g. of raw tissue from New York dressed and eviscerated birds respectively. There was a gradual decrease in the residuals recovered for approximately one month of storage at 3° C.; after this time no antibiotic was detectable.

In the present work, a limited number of assays (10 for CTC and 5 for OTC) were made on macerated skin during storage. Numerically, the results were similar to those obtained by absorption of surface fluids into an assay pad. One difference was that during the first 24-48 hours of storage the values were significantly higher from the absorption of surface fluids than from the macerated skin. However, as storage was prolonged, the two values approached equality which could be attributed to diffusion of the antibiotic throughout the skin.

Figure 2 shows the levels of tetracyclines found in the deep tissue after treatment with solutions containing 30 ug./ml. of the respective antibiotics for 10 minutes and for 120 minutes. When the poultry tissue was treated with these solutions for 10 minutes, the residual in the deep tissue by the 14th

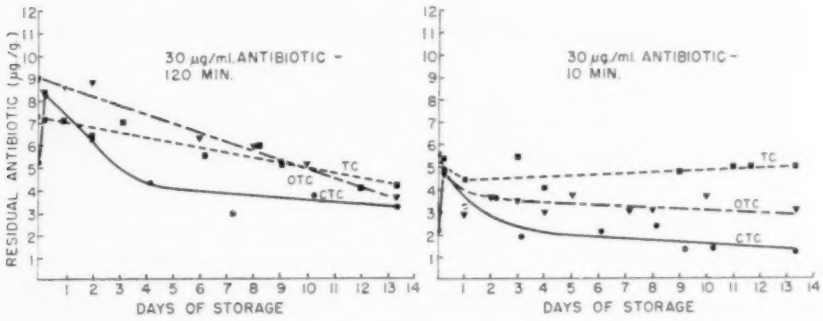

Figure 2. Residuals of tetracyclines in subsurface tissue of antibiotic treated poultry stored at 4° C.

day was appr imately 1.5, 3.0 and 4.5 μg./g. for CTC, OTC and TC respectively; with treatment for 120 minutes, the residuals were approximately 3.5, 4.0 and 5.0 μg./g. respectively. Several experiments continued for 21 days showed that levels of antibiotics decreased only slightly below those found at 14 days. A comparison of the levels of antibiotic found in the tissue after treatment for 10 and 120 minutes illustrates the effect of length of time on antibiotic uptake.

Figure 3 compares the levels of antibiotic found in the 3 different tissues after treatment with CTC and OTC. The treatment of 30 μg./ml. for 120 minutes was selected as being representative of the various treatments.

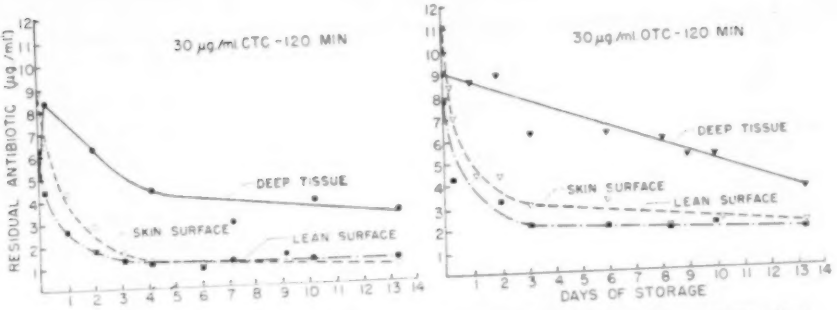

Figure 3. Comparison of levels of chlortetracycline (CTC) and oxytetracycline (OTC) found in different tissues (μg./g. for deep tissue).

Similar relationships were observed for the other treatments but at decreased levels. The antibiotics maintained a high level of activity for a longer period of time in the deep tissue than on the surface tissues. Since the method of assay measures only that antibiotic which is biologically active, the antibiotic in the deep tissue probably was afforded some protection from inactivation. Perhaps on the surface the antibiotic was inactivated by enzymes and metabolic products of bacterial growth or by nonbiological inactivation such as oxidation which would take place rapidly on the surface. Also, part of the rapid decrease of residuals on the surfaces could be attributed to migration and dissipation of the antibiotic to the inner tissues.

In studies on the development of resistance by the microbial flora, preliminary work was done to determine the effect of the culture medium and the temperature of incubation on the stability of the antibiotic. Price, Randall and Welch *(12)* have demonstrated that at 37° C., CTC was unstable in water and that this instability was increased in the presence of a variety of compounds and media. Since CTC was the least stable of the 3 tetracyclines, it was chosen for the determination of a desirable incubation temperature and medium. Addition of agar to the media exerted little influence on the stability of CTC. Penassay broth, nutrient broth and trypticase soy broth were compared; nutrient agar was selected as a suitable medium and 15° C. as the temperature of incubation.

Work reported previously by Ayres *et al. (3)* indicated that organisms of the *Pseudomonas-Achromobacter* group were predominant and developed rapidly on both control and antibiotic treated birds. No indication was given at that time as to the antibiotic susceptibility of the microbial populations occurring on these birds. Ng *et al. (11)* have reported that 63 to 78% of the population on birds exposed to CTC during processing or in laboratory experiments were resistant to 5 p.p.m. CTC, whereas 5 p.p.m. CTC inhibited most of the organisms occurring on unexposed poultry.

Table 1 indicates the changes in percent of organisms susceptible to CTC on both control birds and antibiotic treated birds during storage. On antibiotic treated birds, there was a decided decrease in the percent of organisms inhibited by CTC. On birds treated with a solution containing 10 μg./ml. of

TABLE 1

Percent of total population inhibited by increasing levels of CTC, OTC and TC.

Antibiotic used	Days of storage	Controls				Treated			
		μg antibiotic/ml. nutrient agar				μg antibiotic/ml. nutrient agar			
		0.5	1.0	2.5	5.0	0.5	1.0	2.5	5.0
CTC									
	0	45	75	90	95	58	74	87	90
	2	49	64	72	88	34	39	41	43
	10	53	75	87	87	3	4	5	9
OTC									
	0	67	74	79	88	40	57	69	72
	12	35	51	64	76	5	16	21	24
TC									
	0	65	78	79	86	42	48	56	62
	12	57	64	70	79	8	11	14	17

CTC, 5 μg. CTC added to each ml. nutrient agar inhibited 90% of the organisms on the first day, by the second day 43% were inhibited and by 12 days 9%. Similar findings of increased resistance were found in the presence of OTC and TC.

A comparison of the percentages of organisms inhibited on the control and treated birds on the last day of sampling shows that with 5.0 μg. CTC per ml. of nutrient agar that an average of 87% of the organisms were inhibited but on the treated birds only 9% were affected by the antibiotic (Table 1). After 6 or 8 days there was very little increase in the percentage of organisms that developed resistance to the antibiotic. Final samples were obtained at 10 to 12 days when spoilage was evident and the total surface population was considered to be approaching a maximum. On beef treated with CTC, Jay, Weiser and Deatherage *(8)* found that with the exception of the micrococci, there was little difference in resistance between organisms from control rounds and treated rounds.

A certain number of organisms on the control birds was resistant to the antibiotics. This would suggest that a portion of the normal flora of untreated poultry was resistant to the tetracyclines and that in the presence of the residuals in the poultry meat this resistant flora multiplied and predominated. In addition to resistance to the individual antibiotics, in general, those organisms which were resistant to one of the tetracyclines also were resistant to the other two. Bohonos *et al. (4)*, in studies on the antibacterial spectra of tetracyclines, observed that organisms which were resistant to CTC generally were resistant to TC and OTC. The significance of the development of resistant organisms in processing plants regularly using antibiotics has not been determined.

SUMMARY

Chlortetracycline, oxytetracycline and tetracycline were found to persist in raw tissue at concentrations dependent upon the level of antibiotic used for treatment and the length of time of exposure to the antibiotic. Organisms isolated from poultry treated with CTC, OTC or TC were more resistant to these antibiotics than were those occurring on control birds. In general, organisms resistant to one of the tetracyclines also were resistant to the other two.

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THE SELECTIVITY OF AUTOCLAVE-STERILIZED VIOLET RED BILE AGAR[a]

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It has been stated that "so-called 'typical' colonies appearing in solid media pour plates . . . may not be accepted as colonies of *Escherichia-Aerobacter* unless a sufficiently thorough examination of a large number of colonies from representative samples has proved that they may be so accepted" *(7)*.

Autoclave-sterilization of Violet Red Bile (VRB) Agar in aliquots has been widely practiced because of the convenience in preparing media well ahead of use. Although this practice has been accepted as a standard procedure (*8*, page 144), manufacturers of the medium still suggest *(2, 3)* that VRB Agar be prepared immediately prior to use by heating to boiling. The literature lacks investigations regarding the selectivity of VRB Agar for the enumeration of coliforms in non-dairy foods. Also, little information is available concerning the effects of autoclave-sterilization and re-melting of VRB Agar on the efficacy of the medium. It was considered advisable to examine the selectivity of VRB Agar as affected by the type of food being examined and by the method of preparation of the medium.

MATERIALS AND METHODS

A 100 g. sample of a frozen pot pie or dinner was weighed into a sterile, tared blender jar, 400 ml. of sterile water were added, the mixture was blended for two min., then 1 ml. of the 1:5 dilution was added to each of duplicate sterile petri plates. About 15 ml. of melted, cooled agar were added to each plate, the sample was mixed, the agar was allowed to solidify and plates were overlayed with about 5 ml. of sterile melted agar. The plates were observed after an incubation period of approximately 24 hours at 37° C.

The media used in the following studies were prepared by either of 2 methods. "Autoclaved" agar was prepared in batches of 6-8 liters, melted, bottled in approximately 100 ml. aliquots, and sterilized in the autoclave at 121° C. for 20 min. The bottles of agar were removed from the autoclave as soon as possible following sterilization in order to avoid prolonged exposure to high temperatures. Sufficient quantities were melted for use in the autoclave at 121° C. for 10 min., as needed. "Boiled" medium was prepared immediately before use by heating to boiling to melt the agar and then cooling to 45° C. *(2, 3)*.

Colonies observed on the agar plates were divided into 2 categories: purplish-red colonies 1 or more mm. in diameter were termed "typical" colonies, while purplish-red colonies of less than 1 mm. diameter were called "small" colonies *(3)*.

RESULTS AND DISCUSSION

Parallel coliform tests were made on a series of 40 chicken pot pies (6 different brands) using "boiled" and "autoclaved" VRB Agars. The counts (ranging from less than 3 to 15 per gram) of 27 coliform-positive samples (Table 1) showed that the method of preparation of the agar had little effect

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TABLE 1

Counts obtained on VRB agar prepared by two different methods [1]

	"Boiled"	"Autoclaved"
Number of samples with highest count	8	8
Number of samples with same count	11	11
Total colonies observed on all plates	52	40

[1] See text for method of preparation.

on the counts obtained. Although pies from different processors might be expected to contain different flora (strains of coliforms), performance of the "boiled" and "autoclaved" agars appeared to be similar, regardless of the brand of pie examined. The results do not imply that VRB Agar may be grossly mistreated. Distinct darkening of the agar and inhibitory effects were noted when VRB Agar which had been left to cool in the autoclave overnight following sterilization was used for the coliform count.

A comparison of "autoclaved" VRB and Deoxycholate Agars on 86 poultry and beef pot pies [b] indicated (Table 2) that the 2 media yielded approximately equivalent counts. The average count (count/g./sample) was 28.7 on VRB Agar and 30.6 on Deoxycholate Agar.

TABLE 2

A comparison of VRB and deoxycholate agars for the coliform count of pot pies

Coliforms/g.	Number of Samples (86 total)	
	VRB agar	Deoxycholate agar
Less than 3	26	22
3 to 9	18	24
10 or more	42	40
No. of samples with highest count	30	28
Count/g./sample	29	31

The 2 media were also examined for selectivity. "Typical" colonies were picked from the plates, streaked for isolation on the same medium from which they had been taken, then were tested for lactose fermentation and IMVIC reactions *(6)*. Of 61 "typical" colonies examined (Table 3), 55 (90%) belonged to the *Escherichia* and *Aerobacter* genera (41 *Escherichia spp.*[c], 6 *Aerobacter spp.* and 8 intermediates). In a parallel test, of 42 colonies from plates of raw milk samples, only 12 (29%) belonged to the *Escherichia* and *Aerobacter* genera (4 *Escherichia spp.*, 3 *Aerobacter spp.* and 5 intermediates). Thus, it would appear that the term "coliform count" when applied to frozen pot pies and related products falls well within the definition of "coli-

[b] Twenty-nine chicken pies, 28 turkey pies and 29 beef pies were examined. All samples were obtained in the spring of 1956. No more than 3 samples came from any one lot. Only "typical" colonies were counted.

[c] None of the 41 *Escherichia spp.* isolated could be identified as belonging to serological groups which have been associated with infantile enteropathogenic conditions. It may be concluded that the incidence of these types in frozen pot pies is at least no greater than in milk *(5, 9)*.

form count" as accepted by the dairy bacteriologists (cf.: 4 for recent literature review, studies and discussion regarding dairy products).

Occasionally pot pies may be found which yield low coliform counts as determined by counting "typical" colonies; yet the pies contain relatively large numbers of bacteria which form "small" reddish colonies on VRB Agar plates. Supposedly, red colonies should not be included in the coliform count unless they exceed 0.5 mm. *(8)* or 1-2 mm. *(3)* in diameter. In order to learn the identity of the "small" colonies, 51 "small" colonies (red colonies less than 1 mm. in diameter) were streaked for isolation on Eosin Methylene Blue Agar from VRB Agar plates of pie and dinner samples selected at random. Isolated cultures were carried on Stock Culture Agar during partial identification (Table 4). Thirty-six cultures (71%) belonged to the *Escher-*

TABLE 3

The identity of cultures isolated from "typical" colonies on VRB and deoxycholate agar plates

Type of culture	Percentage of cultures (61 total)	
	VRB agar	Deoxycholate agar
Escherichia spp.	60	74
Aerobacter spp.	17	3
Intermediates	13	13
Non-lactose fermentors	10	10
Number of cultures studied	31	30

TABLE 4

The identity of fifty-one cultures isolated from "small" colonies on VRB agar plates

Identity	% of cultures
Escherichia coli	29
Aerobacter aerogenes	12
Aerobacter cloaceae	2
Intermediates [1]	27
Proteus sp.	2
Actinomycetales	2
Unidentified [2]	26

[1] Included were 3 indole negative *E. intermedium*; 1 indole positive, 3 VP negative and 1 citrate negative *A. aerogenes*; and 6 gelatinase negative *A. cloaceae (1)*.

[2] Seven of the 13 cultures probably belonged to the genera *Alcaligenes* and *Achromobacter (1)*. Five cultures which would be listed as suspected pathogens by ordinary diagnostic procedures were negative when tested with Lederle Salmonella polyvalent diagnostic serum and Shigella grouping sera (groups A, B, C, D and Alkalescens-Dispar).

ichia and *Aerobacter* genera (15 *Escherichia coli*, 6 *Aerobacter aerogenes*, 1 *A-cloaceae* and 14 intermediates). Percentagewise, only about half as many "small" colonies (Table 4) as "typical" red colonies (Table 3) were confirmed to belong to the genus *Escherichia*. Thus, confirmation of *Escherichia* became more urgent when "small" red colonies were present than when only "typical" colonies appeared on the VRB Agar plates. Streak plates of Eosin Methylene Blue Agar were about 90% effective for presumptive identification of *Escherichia* from VRB Agar, when compared with isolation followed by IMVIC tests.

CONCLUSIONS

Violet Red Bile Agar may be sterilized in the autoclave (121° C. for 15-20 minutes) and re-melted (121° C. for 10 minutes) prior to use without substantially affecting the counts obtained from meat pies.

All of the red colonies appearing on VRB Agar plates after 24 hours incubation at 37° C. should be included in the coliform count, regardless of the size of the colony.

The presence of red colonies on VRB Agar plates of frozen pot pies and frozen dinners cannot always be interpreted to indicate the presence of *Escherichia* or closely related genera. A streak plate of Eosin Methylene Blue Agar may be used whenever circumstances dictate confirmation of *Escherichia*.

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STUDIES ON THE LONG-TERM FEEDING OF ACETOGLYCERIDES TO RATS

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Acetylation of natural fats and oils by interesterification, acetylation of superglycerinated fats, or by the acetylation of purified monoglycerides of long chain fatty acids, such as stearic or oleic acids, has resulted in the synthesis of new type fats with usual properties and of potential value as edible fats. These modified fats have been referred to as "acetoglycerides" *(19)*, or "acetin" fats *(5)*. As used here the term "acetoglycerides" refers to both aceto-oleins and acetostearins. To distinguish between the two, the acetylated monoglycerides derived from highly hydrogenated lard or vegetable oils are referred to as acetostearins, and those derived from unsaturated fats as aceto-oleins. These acetoglycerides are undoubtedly the same type as those referred to as "acetylated monoglycerides" by Herting *et al. (21)*.

There is no apparent reason to suppose that acetoglycerides should be hydrolyzed differently *in vivo* than natural fats. There is also no reason to suppose that the metabolic end products should be more toxic than those arising from the metabolism of fats normally present in food. Partial hydrolysis of triglycerides to mono- and diglycerides has been reported to take place during baking and during digestion in man and experimental animals *(6, 23, 24, 27, 29)*. The nutritive value of these mono- and diglycerides has been shown to correspond to that of natural triglycerides of similar fatty acids *(11, 26)*, and the mode of digestion and nutritive value of acetylated monoglycerides have been shown to be the same *(21)*. The rate of absorption of the acetoglycerides was found by Ambrose and Robbins *(1)* to be as good or better than that of a partially hydrogenated shortening (Crisco), while the digestibility of acetostearins and aceto-oleins was comparable to that reported by others *(4, 13, 16, 22)* for completely hydrogenated and unhydrogenated fats. No difference was observed by Ames *et al. (2)* or Bourke and associates *(12)* in the nutritive value of purified monoglycerides or mixtures of mono- and diglycerides when compared with triglycerides.

Triacetin in amounts up to 55% of the diet of rats has been reported to be non-toxic, to support good growth, and to be converted to long chain fatty acids *(15)*. Acetic acid is rapidly utilized when fed to mammals *(18, 25)*, and has been described as occupying a key position in the metabolism of fats, carboyhydrates and certain amino acids *(7)*. By the use of labeled acetate it has been demonstrated that acetic acid disappears rapidly from the blood *(14)*, and labeled carbon dioxide appears in the expired air *(31)*. Acetic acid has been shown to take part in the biosynthesis of cholesterol in man *(20)* and animals *(8)*, in the synthesis of fatty acids *(30, 32)* and in the acetylation of amines *(3, 9, 10)*.

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In the light of this brief review it is difficult to visualize any toxicity resulting from the ingestion of acetoglycerides, particularly since the hydrolytic products, acetic acid, glycerol, free fatty acid, and monoglyceride all occur in the normal course of digestion and metabolism of fats, or are used as such in foods. Nevertheless, the acetoglycerides are new type fats and since they might enter the human diet in appreciable amounts, their investigation for possible chronic toxicity is a Federal Food and Drug Administration requirement.

EXPERIMENTAL

Weanling male and female albino rats of the Slonaker strain, weighing 43 to 51 g., were placed on an adequate basal diet, and on the same diet suitably modified to permit incorporation of acetoglycerides (AG). Table 1 provides information on the identity, and physical and chemical characteristics of the acetoglycerides studied. Since the various acetoglycerides were fed at levels of 5, 10, and 20% of the total diet, it was necessary to make minor adjustments in the composition of the diet to permit maintenance of protein levels and approximately the same caloric value per 100 g. of diet. The composition of the control and acetoglyceride-containing diets is given in Table 2. Two other supplements added to the basal control diet were also fed: (a) 3.3% triacetin and, (b) 6.1% sodium acetate containing 3 molecules of water of crystallization. On a theoretical basis each of these supplements would provide acetate ion equivalent to the amount derived from a diet containing 10% acetoglyceride.

TABLE 2

Percentage composition of diets

Components	Diet groups			
	1	2	3	4
Casein	10	12	11	10
Corn meal (degerm.)	72	50	61	67
Linseed oil meal	10	10	10	10
Alfalfa (ground)	2	2	2	2
Bone ash	1.5	1.5	1.5	1.5
Sodium chloride	0.5	0.5	0.5	0.5
Cod Liver oil (U.S.P.)	3.0	3.0	3.0	3.0
Brewers' yeast	1	1	1	1
Acetoglycerides	0	20	10	5
Protein percent	17.6	17.6	17.6	17.6
Calories/100 grams	360	469	414	388

Thirty rats of each sex were used on each of the acetoglyceride diets, 10 rats of each sex being placed on each of the 3 dietary levels of acetoglyceride. Five female rats were placed on each of the 2 diets containing triacetin and sodium acetate. For control purposes, 30 rats of each sex were placed on the basal diet (diet No. 1, Table 2). The rats were caged in groups of five in solid-bottom cages with wood shavings as litter. Free access to water and to the respective diets was permitted at all times. Food consumption for each group and the body weight of each rat in the group were recorded at regular weekly intervals during the first 300 days, only body weights being recorded thereafter at monthly or bimonthly intervals.

All rats on diets containing 20% of an acetoglyceride and an appropriate number of control rats were sacrificed at the end of 400 days, while rats on diets containing 5 and 10% acetoglyceride sample AG-194 were sacrificed at 600 days; all surviving rats on the 5 and 10% levels of the other acetoglycerides were sacrificed at the end of approximately 709 days. At time of sacrifice all organs were examined for gross evidence of injury. Livers, kidneys, spleens, hearts, and testes were removed, weighed and preserved in a 10% solution of neutralized formaldehyde for histopathological study. In addition, the following tissues were also preserved for histopathological study: bladder, stomach, intestine, pancreas, adrenal, thyroid, brain, hypophysis, parathyroid, ovaries, and uterus. Paraffin sections stained with hematoxylin-eosin were prepared, and in some instances frozen sections of liver were stained with Sudan IV.

TABLE 1

Description, physical and chemical characteristics of acetoglycerides (AG)[1]

Sample No.	Description	Hydroxyl value (free)	Sapon. value	Iodine value	Acetyl %	Linoleic acid %	FFA as oleic acid %	Unsaponifiable matter %	M.P. °C
AG-194	Fully acetylated acetostearin from hydrogenated lard	0.00	389	0.69	16.2	0.04	0.56	0.54	30.7
AG-26	Fully acetylated acetostearin from hydrogenated lard	0.8	380	0.96	18.5	0.13	1.14	0.30	32.5
AG-31	Partially acetylated aceto-stearin from hydrogenated lard	118.4	297	1.08	11.5	0.15	0.61	0.32	57.0
AG-21	Fully acetylated aceto-olein from prime steamed edible lard	0.4	401	42.0	19.8	6.01	0.92	0.23	5.0
AG-3	Fully acetylated aceto-olein from cottonseed oil	0.4	391	73.2	17.9	32.92	0.72	0.59	−2.0

[1] Data supplied by the Southern Utilization Research and Development Division, U. S. Department of Agriculture.

Studies on the effects of the acetoglycerides on reproduction were conducted on each of the 5 acetoglycerides. For this purpose 10 female and 5 male weanling rats were placed on a diet containing 10% of an acetoglyceride, and a similar number of rats of both sexes were placed on the basal diet (diet No. 1, Table 2). After approximately 3 months on the respective diets, all rats were mated and the number of siblings cast and weaned was recorded. Those acetoglycerides permitting successful reproduction in the first generation were studied on several successive generations. All rats receiving other acetoglycerides were remated several times before discontinuance.

RESULTS

Longevity. The survival data are summarized in Table 3. They indicate that none of the acetoglycerides even at the 20% level altered mortality during the first 400 days. Rats on 20% were not carried beyond 400 days. On the 5 and 10% levels most of the deaths occurred during the last 100 days of the 709-day test period. The greatest change in survival occurred in male and female rats receiving acetoglyceride sample AG-26, and in male rats receiving samples AG-21 and AG-3. Results obtained with diets containing triacetin or sodium acetate suggest that the acetate ion that might be derived from a 10% acetoglyceride diet did not contribute to an increased mortality.

Growth. The data on body weights are summarized in Table 4. With the possible exception of male rats receiving 20% of samples AG-194 and AG-31, and female rats receiving 20% of sample AG-31 there were no marked effects on growth rate and final body weights.

Organ weights. Tables 5 and 6 summarize the data on organ weights. As shown in Table 5, the only organs consistently affected by the 20% level of the various acetoglycerides were the testes and liver. The testes of rats on the 3 acetostearin diets (AG-194, AG-26, AG-31) were significantly smaller than those of the control rats or rats on the two aceto-olein diets (AG-21 and AG-3). The livers of male rats on 20% AG-194 and of both male and female rats on 20% AG-31 were significantly larger than those of control rats. Although the total ether extractives (fat) from these livers did not indicate fat storage, fat droplets were observed microscopically. The livers of female rats on 20% AG-21 and AG-3 were smaller than those of control rats, showed increased amounts of ether extractives, and exhibited fat droplets microscopically. Kidneys of female rats on 20% AG-31 were significantly larger than those of the controls.

Data on the 5 and 10% levels of the acetoglycerides for 600 days or more show no real differences between control and experimental groups except for a decrease in weights of testes of rats receiving AG-194 for 600 days or AG-31 for two years.

Liver fat and glycogen. In Table 7 data are summarized on liver fat and glycogen for rats on 20% acetoglyceride diets for 400 days. Although the liver fat values were consistent within experiments, considerable variation was encountered between the different acetoglycerides. Liver fat values for the males on the 3 acetostearins (AG-194, AG-26, AG-31) were slightly lower than those of the controls whereas the liver fat values for the 2 aceto-oleins (AG-21 and AG-3) were considerably higher. In the females no marked difference was observed between the controls and the experimental groups, except for the group of rats on AG-3. The actual liver weights in g./100 g. of body weight (Table 5)had no relationship to the fat found, and histologically no pathological changes were observed.

Liver glycogen values for both male and female rats, summarized in Table 7, suggest that the acetoglycerides were devoid of glycogenic activity. Where changes in liver glycogen were observed the values were always below those found for control rats on diet 1. These observations on liver glycogen storage after the ingestion of diets containing 20% acetoglycerides for 400 days are comparable to results reported on shorter experiments for natural fats and oils *(17)*, and for acetic acid *(28)*.

Efficiency of food utilization. The effect of the acetoglyceride diets on the efficiency of food utilization over a 100-day period is evident from the data summarized in Table 8. In general the acetostearins decreased the efficiency more than did the aceto-oleins, and the effect was related directly to the concentration in the diet.

TABLE 3

Survival data for rats on diets containing acetoglycerides

Supplements fed	Dietary conc. %	Number of survivors (days)							
		0	100	200	300	400	500	600	700
Males									
Controls	0	30	28	28	28	27[1]	19	18	14
AG-194	5	10	10	10	10	7	7	6	....
	10	10	10	10	9	8	5	5	....
	20	10	10	10	9	9	....	....	....
AG-26	5	10	10	10	10	9	9	9	4
	10	10	10	9	9	9	9	9	4
	20	10	8	8	8	8	....	....	....
AG-31	5	10	9	9	9	9	9	8	8
	10	10	8	8	7	7	7	6	6
	20	10	7	7	7	7	....	....	....
AG-21	5	10	10	10	10	10	10	9	5
	10	10	10	10	9	9	9	8	4
	20	10	9	9	9	9	....	....	....
AG-3	5	10	10	10	10	9	9	9	3
	10	10	8	8	7	5	5	5	2
	20	10	10	10	10	9	....	....	....
Females									
Controls	0	30	29	29	29	28[1]	18	17	11
AG-194	5	10	10	10	10	10	4	4	....
	10	10	10	10	10	10	8	8	....
	20	10	10	10	9	9	....	....	....
AG-26	5	10	9	9	9	8	7	5	4
	10	10	10	10	10	9	8	7	3
	20	10	9	9	9	9	....	....	....
AG-31	5	10	10	9	8	8	8	8	7
	10	10	10	10	10	10	10	9	8
	20	10	7	7	6	6	....	....	....
AG-21	5	10	10	10	10	10	10	10	8
	10	10	10	10	10	9	8	7	5
	20	10	9	9	8	8	....	....	....
AG-3	5	10	10	10	10	10	10	8	7
	10	10	10	10	10	10	10	8	6
	20	10	10	10	10	9	....	....	....
Triacetin	3.3	5	5	5	5	5	3	3	3
Sodium Acetate	6.1	5	5	5	5	5	5	5	5

[1] Seven male and eight female rats were sacrificed at 400 days.

TABLE 4

Growth data for rats on control and acetoglycerides supplemented diets

Supplements fed	% In diet	Average body weight at approximate time interval indicated (days)							
		0	50	100	200	300	400	600	700
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Males		50	219	297	353	381	415	.....	.....
Control	0							424	413
AG-194	5	50	226	307	376	394	414	456	.....
	10	51	229	309	365	386	390	412	.....
	20	49	190	270	335	353	361	.....	.....
AG-26	5	49	229	304	335	381	405	429	428
	10	50	228	316	347	397	405	435	446
	20	49	204	281	326	367	393	.....	.....
AG-31	5	50	190	260	316	358	378	396	373
	10	50	185	240	292	352	370	398	385
	20	50	128	201	255	299	328	.....	.....
AG-21	5	49	211	290	333	376	398	417	410
	10	49	230	324	369	420	435	450	472
	20	50	203	291	326	368	393	.....	.....
AG-3	5	49	214	301	369	399	427	435	455
	10	49	195	292	360	392	426	452	447
	20	49	190	287	368	386	405	.....	.....
Females		43	160	205	252	277	306	.....	.....
Control	0							332	341
AG-194	5	43	157	201	245	265	271	339	.....
	10	43	167	207	255	277	297	352	.....
	20	44	154	197	229	274	288	.....	.....
AG-26	5	44	167	213	260	278	312	362	365
	10	44	160	204	251	257	305	321	323
	20	45	155	205	249	261	292	.....	.....
AG-31	5	43	161	205	255	279	299	337	340
	10	43	148	204	253	273	305	362	356
	20	43	130	189	253	247	251	.....	.....
AG-21	5	43	169	212	252	270	279	334	340
	10	44	163	215	258	283	311	348	358
	20	44	152	206	250	269	300	.....	.....
AG-3	5	43	154	213	282	310	337	365	378
	10	43	153	205	263	286	320	353	363
	20	44	161	219	282	307	345	.....	.....
Triacetin	3.3	49	169	208	244	286	293	305	313
Sodium Acetate	6.1	49	179	216	254	278	292	313	304

Reproduction. Rats on diets containing 10% aceto-olein (AG-3 and AG-21) supported reproduction and lactation through the test period of three generations. Each generation of rats was remated several times, and the rats remained fertile throughout. The total litters cast, the number of siblings per litter and the average body weights at weaning, and at 50 and 100 days for each generation were similar in the control and experimental groups. With the acetostearins, AG-194, AG-26, and AG-31, only AG-26 supported reproduction through 3 generations comparable to the controls. Both AG-194 and AG-31 failed to support reproduction beyond the first generation.

Gross pathology. On post-mortem examination no gross changes were seen that were not also seen in control rats, such as respiratory infection,

TABLE 5

Body and organ weights of rats on control and acetoglyceride supplemented diets for 400 days

Supplements fed	% in diet	No. of rats	Body weights grams	Organ weights (grams/100 body weight)				
				Liver	Kidneys	Testes	Spleen	Heart
			Mean ± S.E. [1]	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
Males								
Control	0	8	418 ± 12.1	2.87 ± 0.08	0.55 ± 0.06	0.83 ± 0.05	0.18 ± 0.01	0.29 ± 0.01
AG-194	20	9	361 ± 7.7 *	3.54 ± 0.08 *	0.64 ± 0.01	0.50 ± 0.05 *	0.21 ± 0.08	0.38 ± 0.02
AG-26	20	8	393 ± 9.4	2.85 ± 0.02	0.57 ± 0.0	0.66 ± 0.01 *	0.16 ± 0.00	0.27 ± 0.01
AG-31	20	7	324 ± 19.7 *	3.41 ± 0.16 **	0.68 ± 0.08	0.41 ± 0.03 *	0.18 ± 0.03	0.29 ± 0.01
AG-21	20	9	403 ± 11.2	2.60 ± 0.08	0.52 ± 0.02	0.81 ± 0.01	0.14 ± 0.01	0.27 ± 0.02
AG-3	20	9	406 ± 9.7	2.72 ± 0.01	0.58 ± 0.01	0.78 ± 0.01	0.18 ± 0.02	0.30 ± 0.01
Females								
Control	0	9	291 ± 10.0	3.13 ± 0.05	0.53 ± 0.01		0.16 ± 0.0	0.30 ± 0.0
AG-194	20	9	288 ± 7.4	3.21 ± 0.03	0.61 ± 0.03		0.20 ± 0.0	0.35 ± 0.01
AG-26	20	9	290 ± 8.8	3.41 ± 0.04	0.66 ± 0.01		0.21 ± 0.01	0.32 ± 0.01
AG-31	20	6	251 ± 17.6	3.69 ± 0.14 *	0.90 ± 0.13*		0.23 ± 0.04	0.35 ± 0.03
AG-21	20	8	300 ± 9.8	2.75 ± 0.08 *	0.53 ± 0.01		0.16 ± 0.01	0.28 ± 0.01
AG-3	20	9	344 ± 6.9	2.59 ± 0.08 *	0.49 ± 0.01		0.16 ± 0.01	0.26 ± 0.0

[1] S.E.—Standard Error of the Mean
* = 0.001
** = 0.01

TABLE 6

Body and organ weights on control and acetoglyceride supplemented diets for approximately 2 years

Supplements Fed	% in diet	No. of rats	Days on diets	Bd. wts. grams Mean ± S.E. [1]	Organ weights (gram/100 grams body weight)				
					Liver	Kidneys	Testes	Spleen	Heart
					Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
Males									
Control	0	11	709	408 ± 12.4	2.65 ± 0.13	0.65 ± 0.02	0.80 ± 0.02	0.17 ± 0.01	0.29 ± 0.01
AG-194	5	6	600	456 ± 17.9	2.63 ± 0.15	0.58 ± 0.01	0.49 ± 0.07	0.17 ± 0.01	0.28 ± 0.01
	10	5	600	411 ± 26.5	3.16 ± 0.11	0.63 ± 0.02	0.45 ± 0.11	0.19 ± 0.02	0.30 ± 0.02
AG-26	5	4	709	389 ± 38.2	2.70 ± 0.10	0.59 ± 0.03	0.86 ± 0.10	0.19 ± 0.03	0.29 ± 0.01
	10	4	709	451 ± 8.6	2.53 ± 0.11	0.59 ± 0.02	0.70 ± 0.05	0.16 ± 0.01	0.26 ± 0.01
AG-31	5	5	710	354 ± 23.7	2.62 ± 0.23	0.66 ± 0.05	0.26 ± 0.04	0.20 ± 0.03	0.32 ± 0.01
	10	4	711	387 ± 18.4	2.95 ± 0.21	0.63 ± 0.01	0.22 ± 0.02	0.20 ± 0.02	0.29 ± 0.03
AG-21	5	5	709	405 ± 20.2	2.67 ± 0.05	0.64 ± 0.01	0.79 ± 0.01	0.16 ± 0.01	0.27 ± 0.01
	10	4	709	454 ± 18.0	2.45 ± 0.12	0.54 ± 0.02	0.79 ± 0.02	0.15 ± 0.02	0.27 ± 0.02
AG-3	5	3	709	395 ± 11.1	2.86 ± 0.22	0.64	0.87	0.16 ± 0.32	0.38
	10	1	713	402	4.08	0.68	0.78	1.09	0.32
Females									
Control	0	10	707	301 ± 19.0	3.65 ± 0.26	0.68 ± 0.03		0.31 ± 0.05	0.34 ± 0.01
AG-194	5	4	600	338 ± 12.7	2.90 ± 0.18	0.55 ± 0.04		0.26 ± 0.05	0.27 ± 0.04
	10	8	600	353 ± 8.2	2.83 ± 0.10	0.54 ± 0.01		0.18 ± 0.01	0.28 ± 0.03
AG-26	5	4	707	372 ± 19.0	2.93 ± 0.17	0.55 ± 0.02		0.17 ± 0.02	0.28 ± 0.02
	10	3	707	339 ± 6.4	3.19 ± 0.22	0.63 ± 0.04		0.30 ± 0.01	0.34 ± 0.01
AG-31	5	6	714	318 ± 16.9	2.99 ± 0.23	0.61 ± 0.02		0.16 ± 0.01	0.30 ± 0.02
	10	5	714	315 ± 13.5	2.95 ± 0.14	0.61 ± 0.02		0.19 ± 0.01	0.32 ± 0.02
AG-21	5	7	707	335 ± 11.2	2.80 ± 0.11	0.53 ± 0.02		0.19 ± 0.02	0.30 ± 0.02
	10	5	707	335 ± 6.0	2.54 ± 0.12	0.52 ± 0.01		0.15 ± 0.01	0.27 ± 0.01
AG-3	5	5	715	361 ± 16.4	3.13 ± 0.13	0.57 ± 0.03		0.20 ± 0.01	0.31 ± 0.02
	10	6	715	343 ± 22.5	3.09 ± 0.18	0.57 ± 0.02		0.21 ± 0.02	0.31 ± 0.01
Triacetin	3.3	3	708	312 ± 17.1	4.10 ± 0.05	0.65 ± 0.03		0.19 ± 0.01	0.34 ± 0.01
Sodium acetate	6.1	4	708	311 ± 14.3	3.76 ± 0.01	0.65 ± 0.04		0.24 ± 0.03	0.37 ± 0.01

[1] S.E. = Standard Error of the Mean

TABLE 7

Fat and glycogen content of livers from rats on control and acetoglyceride supplemented diets

Supplement fed	% in diet	Days on diet	No. of rats		Body weight Grams		Liver weight Grams		% ± S.E. [1]			
									Fat		Glycogen	
			Male	Female	Male	Female	Male	Female	Males	Females	Males	Females
Control	0	400	8	9	418	291	12.03	9.11	6.34 ± 0.34	6.26 ± 0.49	2.71 ± 0.17	2.33 ± 0.09
AG-194	20	400	9	9	361	288	12.78	9.25	5.28 ± 0.34*	6.49 ± 0.33	2.17 ± 0.37	2.00 ± 0.18
AG-26	20	400	8	9	393	290	11.24	9.90	5.23 ± 0.17*	6.04 ± 0.59	1.86 ± 0.35*	1.93 ± 0.10**
AG-31	20	400	7	6	324	251	11.04	9.28	4.76 ± 0.25*	6.67 ± 1.16	2.05 ± 0.09	1.51 ± 0.26**
AG-21	20	400	9	8	403	300	10.49	8.26	8.19 ± 0.50**	6.73 ± 0.47	1.40 ± 0.02**	2.14 ± 0.19
AG-3	20	400	9	9	406	344	11.05	8.89	10.28 ± 0.68**	8.12 ± 0.53*	1.66 ± 0.24**	2.12 ± 0.01

* = Significant.
** = Highly Significant.
[1] = Standard Error of the mean.

TABLE 8

Body weight increases and efficiency of food utilization for rats on control and acetoglyceride supplemented diets for 100 days [1]

Supplements fed	% in diet	No. of rats	Males: Body weights Start	Males: Body weights End	Males: Body weights Gain	Males: Total food eaten	Males: Efficiency of diet [1]	No. of rats	Females: Body weights Start	Females: Body weights End	Females: Body weights Gain	Females: Total food eaten	Females: Efficiency of diet [1]
Control	0	10	50	290	240	1206	5.53	10	42	201	159	975	4.50
AG-194	5	10	50	306	256	1220	5.48	10	43	202	159	960	4.26
	10	10	51	308	257	1269	4.89	10	43	208	165	1015	3.93
	20	10	49	272	223	1097	4.33	10	44	197	153	917	3.55
AG-26	5	10	49	304	255	1207	5.44	9	44	213	169	932	4.67
	10	10	50	316	266	1246	5.16	10	44	204	160	951	4.06
	20	8	50	281	231	1170	4.21	9	45	205	160	907	3.76
AG-31	5	9		261	211	1135	4.79	10	43	204	161	978	4.24
	10	8	50	240	190	1188	3.86	10	43	202	159	995	3.86
	20	7	51	202	151	845	3.81	7	43	187	144	817	3.76
AG-21	5	10	49	292	243	1123	5.56	10	43	213	170	945	4.63
	10	10	49	324	275	1214	5.41	10	44	216	172	899	4.62
	20	9	50	296	246	1097	4.78	9	44	208	164	848	4.12
AG-3	5	10	49	300	251	1147	5.64	10	43	212	169	867	5.03
	10	8	49	292	243	1122	5.23	10	43	207	164	823	4.81
	20	10	49	283	234	1025	4.87	10	44	222	178	861	4.41
Triacetin	3.3							5	49	208	159	990	
Sodium Acetate	6.1							5	49	217	168	1123	
Control	0	5	45	268	223	1267	4.89						
Crisco	5	5	44	276	232	1306	4.58						
Crisco	10	5	45	286	241	1246	4.67						
Crisco	20	5	48	263	215	1057	4.29						

[1] Efficiency = gain in weight/calories consumed × 100

lung consolidations, adhesions, cysts, middle ear infection, and other infections. Bladder stones were found in one control rat autopsied at 700 days, and in 17 experimental rats, of which only one was a female. The greatest frequency of bladder stones occurred in rats fed 20% AG-31, AG-26, and AG-21 for 400 days, stones being found in 2, 5 and 3 rats respectively. Bladder stones occurred in one rat receiving 5% AG-194 for 600 days, and at the end of 700 days in one rat on 5% AG-31, one rat on 10% AG-26, and in 2 rats each on 5 and 10% AG-21. No bladder stones were found in rats on any dietary level of AG-3.

In at least 50% of the rats on all levels of AG-194, AG-31, AG-26 and AG-21, the uteri were discolored, "rusty." No discoloration of the uteri was observed in the rats in the control group or in rats on all levels of AG-3.

Testes of all rats on the diets containing 20% acetostearins for 400 days, on 5 and 10% AG-194 for 600 days, and 5 and 10% AG-31 for 700 days were considerably smaller than those of the controls. A total of 34 tumors was found and these occurred principally in rats that were 600 to 700 days old. The incidence of tumors was no greater in the experimental groups than it was in the control groups. Only 8 tumors were found between 400 and 600 days on the respective diets, the remaining 26 were found after 600 days.

Microscopic pathology. Sections of liver from control and experimental rats stained for fat showed the presence of more fatty material in the livers of animals fed various samples of acetoglycerides at dietary levels of 20% for 400 days than was seen in control livers. The variability of fat deposition within different animals of the same group was striking. Also, within the same liver the deposition was patchy, so that when some hepatic cells were greatly distorted by deposited lipid, other cells showed none. Similarly affected cells usually occurred in groups situated about central veins, but the distribution of the change within the liver lobules was not constant. The absence of other evidence of liver cell injury suggests that the fatty deposits represent a storage phenomenon. Hematoxylin-eosin stained liver sections showed these changes as vacuolation. Livers of animals receiving lower levels of acetoglycerides for 700 days showed about the same frequency and degree of vacuolation as noted on the 20% level after 400 days. Some of the animals receiving 10% of the various acetoglycerides in the diet for 700 days exhibited a little more irregularity in size of the hepatic cells than was seen in the controls. Occasional clusters of large cells were seen. They were all less than 1 mm. in diameter and their appearance gave no suggestion of malignancy.

A severe degree of testicular atrophy was present in rats receiving dietary levels of 20% AG-194 or AG-31 for 400 days, and the same damage existed in animals on 10% or 5% AG-31 for 700 days. Well-developed testicular atrophy was found in all animals receiving 10% AG-194 for 600 days, whereas the degree of atrophy was moderate in 4 of 6 animals receiving 5% AG-194 for 600 days, testicular structure being practically normal in the other two. The testes of animals receiving acetoglycerides AG-21, AG-26, and AG-3 were not distinctly different from those of the controls.

Kidneys from some animals in different experimental groups showed tiny focal deposits of basophilic material, presumably calcium, commonly associated with irregular tubular dilatation and patchy scarring. This calcification usually appeared within the tubules of the medullary pyramids and was apparently precipitated in the substance of casts. It was sometimes present

also as small lumps in the interstitial tissue, usually in the medulla, and sometimes it occurred as small calculi in the pelvis.

These kidney changes occurred principally in organs from animals which received 20% AG-194, 20% AG-26, and 20% AG-31. Most of these lesions did not distort the kidneys greatly but several of them were severe. The smaller doses of these substances for 600 and 700 days resulted in minor changes of the same type in a portion (less than half) of the animals. AG-21 and AG-3 produced essentially no calcification or scarring of the kidneys at any of the dose levels.

A suggestion of a sex difference could be observed in the kidney lesions noted above. Changes in the female kidneys were most severe after AG-26, while the most prominent lesions in the male kidneys were seen in the AG-194 and AG-31 animals. In each of the affected groups, however, there were some kidneys which showed very little or no alteration.

An interesting alteration of the fatty tissue occurred in many of the treated animals on AG-194, AG-26, and AG-31 for 400 days, but not in the animals on AG-21 or AG-3. Somewhat less prominent similar changes were present after 10% or 5% AG-194 for 600 days, and practically no such changes were found in the 700-day animals.

This lesion consisted of small foci of foreign body reaction in which macrophages and small giant cells were clustered about what appeared to be the residue of fat droplets. Each such structure presented a central space in which there were often fine clefts in a faintly stained matrix, suggesting that fine crystals had been dissolved out during preparation of the specimens. These suggest fatty acid crystals and are consistent with the idea that some focal change in the stored fat has caused it to act as a foreign body. Commonly several adjacent fat cells were similarly involved, producing a clump of fused nodules of foreign body reaction. The adjoining surrounding fatty tisue, however, appeared perfectly normal.

This change was seen most frequently and most prominently in the small amount of fatty tissue attached to the outer surface of the stomach, but it occurred also next to the small intestine, adjacent to the bladder and around the ovary. Occasionally a small focus was related to the spleen, pancreas, adrenal or testis. The reaction was not found around the renal pelvis or in the fatty tissue of the thorax or neck. It should be noted, however, that only small samples of fat were available inasmuch as they were present only incidentally to the preparation of sections of other organs.

The appearance of this lesion brings to mind the disease of human infants known as sclerema adiposum neonatorum, in which the fat of the adipose tissue has an abnormal, sometimes crystalline, structure and excites a foreign body reaction. One of the suggested causes of this disease is a deficiency of olein in the adipose tissue, causing the fat to solidify at a higher temperature than is usual *(33)*.

The other organs of the experimental animals showed no changes which could be clearly distinguished from those in controls. Pneumonia, and inflammation of the endometrium occurred fairly frequently without relation to the treatment. Similarly, several mammary adonomas in older animals and occasional other tumors cannot be ascribed to the treatment.

The animals which received triacetin and sodium acetate were not distinctly different from the controls.

SUMMARY

Three samples of acetostearin and two samples of aceto-olein were fed to albino rats at dietary levels of 5, 10 and 20% for periods ranging from 400 days at the 20% level to approximately 2 years on the lower levels.

No significant differences in growth were noted between the control animals and those fed acetoglycerides except in the case of male rats on dietary levels of 20% acetostearins AG-194 and AG-31, and female rats receiving 20% AG-31.

Testes of rats receiving the three acetostearin diets were significantly smaller than those of the control rats or rats fed the two aceto-oleins. The livers of male rats fed 20% acetostearin AG-194 and of both male and female rats fed 20% acetostearin AG-31 were significantly larger than those of the control rats. Livers of female rats fed 20% of either aceto-olein were smaller than those of the control rats. Kidneys of female rats fed 20% acetostearin AG-31 were significantly larger than those of the controls.

The storage of liver glycogen by rats on the various acetoglyceride-containing diets was not enhanced. Excess fat observed in rats fed the various acetoglycerides appeared to be a storage phenomenon since it was unaccompanied by evidence of cellular damage.

Acetostearins produced a foreign body reaction in the fatty tissue of a number of the organs, presumably due to crystalline deposits of a high melting point fat.

Acetostearins AG-194, AG-26, and AG-31 produced some scarring of kidney tissue, presumably due to deposited calcium.

Acetostearins AG-194 and AG-31 inhibited reproduction. This finding correlates well with the decrease in weight of the testes and the histopathological evidence of testicular atrophy produced by these two acetostearins. It also correlates with the "rusty" discoloration of the uteri observed in at least half of the rats receiving these acetostearins and suggests an increase in vitamin E requirement. The fact that discolored uteri were also seen in rats receiving acetostearin AG-26 and aceto-olein AG-21, and that the former gave rise to small testes without microscopic evidence of atrophy suggest the increase in E requirement was not great enough to interfere with reproduction in these instances.

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EFFECT OF ACETOSTEARIN ON REPRODUCTION IN ALBINO RATS

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The effect of three acetostearins (AG-194, AG-26, and AG-31) and two aceto-oleins (AG-3 and AG-21) on reproduction and lactation in albino rats through a test period of three generations has been reported by Ambrose, Robbins and Cox *(1)*. When acetostearin samples AG-194 and AG-31 were added to an adequate basal ration at dietary levels of 10%, significant decreases occurred in the number of litters cast and the survival of the young. Acetostearin AG-26 permitted normal reproduction and survival on the same dosage level, as did the two aceto-oleins. Not only did reproduction efficiency decrease when both sexes received acetostearins AG-194 and AG-31, but it also decreased when male or female rats receiving acetostearin were crossmated with rats on the control diet. The effects on reproduction were consistent with the "rusty" discoloration of the uteri and decrease in weight of the testes observed at time of autopsy. Table I provides information on the preparation and characteristics of the five acetoglycerides studies by Ambrose, Robbins and Cox *(1)*.

Before proceeding with an analysis of the cause of the effects on reproduction and lactation occurring on diets containing the two acetostearins it should be noted that the effects occurred despite the following circumstances. The rats on the control basal diet reproduced well and weaned the offspring successfully. This fact agrees with our experience over a period of many years with this same diet. The peroxide values for the control diet and the 10% acetostearin diet were 0.0026 and 0.002 meq/g. of diet respectively. The vitamin E content of the control diet and acetostearin diet as determined by the Florisil method was 20 and 25μg/g. of diet respectively. Since the acetostearin contained 0.02% BHA (butylated hydroxy anisole) as an antioxidant, the 5 μg. additional reducing material (apparent vitamin E) per gram of acetostearin diet can be accounted for by the residual BHA in the acetostearin. The true vitamin E content of the two diets may, therefore, be regarded as identical. Moreover, the residual BHA in the acetostearin would be expected to exert a protective action on the vitamin E content of acetostearin diet.

The addition of 10% acetostearin to the basal diet necessitated minor adjustments in the amounts of certain basal constituents to permit maintenance of protein content and approximate caloric value. The composition of the control and experimental diets is given in Table 2. Since reproduction was good on the basal diet, and since no essential difference exists between the

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TABLE 1

Description, physical and chemical characteristics of acetoglycerides (AG) [1]

Sample No.	Description	Hydroxyl value (free)	Sapon. value	Iodine value	Acetyl %	Linoleic Acid %	FFA as oleic acid %	Unsaponifiable matter %	M.P. °C
AG-194	Fully acetylated acetostearin from hydrogenated lard	0.00	389	0.69	16.2	0.04	0.56	0.54	30.7
AG-26	Fully acetylated acetostearin from hydrogenated lard	0.8	380	0.96	18.5	0.13	1.14	0.30	32.5
AG-31	Partially acetylated acetostearin from hydrogenated lard	118.4	297	1.08	11.5	0.15	0.61	0.32	57.0

[1] Data supplied by the Oilseed Section, Southern Utilization Research and Development Division, U. S. Department of Agriculture.

TABLE 2
Composition of diets

Components	Diet Groups	
	1	2
	%	%
Casein	10	11
Cornmeal (degerminated)	72	61
Linseed oil meal	10	10
Alfalfa (ground)	2	2
Bone ash	1.5	1.5
Sodium chloride	0.5	0.5
Cod liver oil (U.S.P.)	3.0	3.0
Brewers' yeast	1	1
Acetoglycerides	0	10
Protein percent	17.6	17.6
Calories/100 g.	360	414

two diets as regards peroxide value, vitamin E content, or fatty acids supplied by cod liver oil, cornmeal and linseed oil meal, one is forced to conclude that the poor reproduction on the acetostearin diet was due to increased requirements for vitamin E and/or essential fatty acids.

The literature dealing with the physiological effects of vitamin E deficiency has been summarized by MacKenzie *(5)*, and the need for essential fatty acids in normal reproduction and lactation has been reported by Kummerow and associates *(4)* and by Deuel *et al. (3)*. Finally Moore *et al. (6)* have reviewed the work of Dam and coworkers and others, and reported independent evidence on some of the beneficial effects of methylene blue in counteracting some of the effects of vitamin E deficiency.

EXPERIMENTAL

The information summarized thus far suggested that supplementation of the diet containing 10% acetostearin, in this case sample AG-194, with alpha-tocopherol, soybean oil as a source of essential fatty acids, alpha-tocopherol plus soybean oil, or methylene blue would throw light on the reproduction effects noted above. The composition of the acetostearin diet is given in column 2 to Table 2. To each kilo of this diet one of the following supplements was added: 200 mg. alpha-tocopherol; 20 g. of soybean oil; 200 mg. alpha-tocopherol plus 20 g. of soybean oil; 1.25 g. of methylene blue.

A sufficient number of weanling male and female rats from our own stock was placed on the acetostearin diet without added supplements. Fifty days later the rats were divided into 4 groups of 5 males and 10 females each, and one group of 4 males and 8 females. The latter group was placed on the acetostearin diet supplemented with methylene blue. One of the other 4 groups was placed on the unmodified acetostearin diet, the other 3 groups being placed on the supplemented acetostearin diets described above. The male and female rats of each group were kept separate on their respective diets until 100 days of age at which time they were mated. All rats were kept on their respective diets for approximately 7 months, during which time they were mated 4 times. Records were kept of the size of litters produced and the number of young weaned on each diet.

RESULTS AND DISCUSSION

The data obtained on reproduction performance are summarized in Table 3.

The 4 successive matings on the diet containing 10% acetostearin desig-

TABLE 3

Reproduction performance on supplemented and nonsupplemented acetostearin diets

Diet	First mating	Second mating	Third mating	Fourth mating
Control	12/9/75/42	11/9/46/25	11/6/34/29	
10% AG 194 [1]	9/9/45/19	8/7/25/14	8/4/17/8	7/1/1/0
10% AG 194 + E + Corn Oil	9/9/57/37	9/8/48/15	9/8/62/43	9/7/41/18
10% AG 194 + E	10/7/39/32	10/8/44/1	10/7/43/18	8/6/30/13
10% AG 194 + Soybean Oil	10/2/5/0	10/0/0/0	10/0/0/0	10/0/0/0
10% AG 194 + Methylene Blue	8/4/16/14	7/5/20/6	7/6/35/13	7/5/19/5

Key: The figures given for each mating refer to — number of females/number of litters/total number of pups/total number weaned.

[1] At time of autopsy brown discoloration of the uteri was observed in all female rats on the diet containing 10% AG 194 with and without the soybean oil supplement. The uteri on all other diets appeared normal in color.

nated as AG-194 clearly reveal a progressive decrease in the number of litters, the total number of pups, and diminishing success in weaning. This suggests that while analysis showed an amount of vitamin E comparable to that in the control diet this amount was inadequate in the presence of the acetostearin and that body stores of vitamin E were depleted in successive matings. In agreement with observations by others the addition of methylene blue eliminated the brown discoloration of the uteri and restored reproduction performance to a significant degree. The addition of vitamin E to the acetostearin diet improved performance and further improvement resulted when both vitamin E and soybean oil were added. The addition of soybean oil alone to the acetostearin diet practically eliminated reproduction. This observation is in harmony with the idea that oxidation of the unsaturated fatty acids facilitates destruction of vitamin E *(2)* and raises requirements for this vitamin.

Collectively, the data suggest strongly that a diet containing 10% acetostearin AG-194 and having a vitamin E content normally adequate for satisfactory reproduction performance failed primarily because of increased vitamin E requirement and not because of a lack of essential fatty acids. Moreover, the beneficial effect obtained by adding the antioxidant methylene blue to the acetostearin diet suggests that the acetostearin raised the vitamin E requirements by facilitating its destruction rather than by interference with its absorption.

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BEHAVIOR OF STARCH DURING FOOD PREPARATION
I. SOME PROPERTIES OF STARCH-WATER SYSTEMS[a]

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Fundamental to understanding the behavior of starch-thickened food products is a more complete characterization of various food starches according to their behavior during food preparation. That starches from different botanical sources differ in certain of their properties has long been recognized. Since Nägeli's observations *(17)* nearly one hundred years ago that potato and wheat starches are affected differently by certain chemicals, numerous later investigators *(5, 7, 23)* have compared some of the physical properties of different starches. The importance of these differences in physical properties to practical applications is well recognized and the variety of natural starches available has been augmented by many chemically modified starches of diverse characteristics.

Despite the many studies of the physical and chemical properties of these various natural and modified starches, recorded data on certain properties of special importance in food products, such as gel strength and cold-paste viscosity, seem to be meager. For this reason, choice of a particular starch for use in a particular food has been largely an art in the past, and the effects of varying the conditions under which it was used have been little understood. The few studies along this line which have been reported serve to point out the value of further work.

Woodruff and her associates *(28, 29)* found that 5% pastes of corn, wheat, rice, potato, arrowroot and cassava starches all failed to form gels unless they were heated above 90° C. although cassava starch gave no gel even after heating to 99.5° C. Whittenberger and Nutting *(26)* and Hofstee and de Willigen *(8)*, however, reported that gel strength of potato starch pastes heated to various temperatures passed through a maximum value, and the latter investigators indicated that maximum gel strength corresponded to maximum hot-paste viscosity. Seck and Fischer *(21)* found no correlation between gel strength and viscosity for a number of different starches, although they and others *(4, 18)* found a relation between viscosity and the degree of swelling of the granules. These and other investigations *(1, 15, 27)* led to the conclusion that gel strength and viscosity are two distinct and different properties of the pastes.

The present series of studies may help to furnish a sounder basis for selecting the best starch for a given use and also elucidate the conditions which will give the most desirable results with the particular starch chosen. The purpose of this study was to determine how the extent of cooking affected a number of properties of starch pastes and to compare a variety of starches and flours in this respect. To this end sixteen representative food starches

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and three flours were cooked in a Corn Industries Viscometer and samples were taken at various points during the cooking. Points at which cooking was stopped and samples were taken for further tests were determined by positions in reference to the maximum viscosity on the gelatinization curve rather than by temperature or length of cooking time alone, since the different starches used had different gelatinization temperature.

EXPERIMENTAL

Materials. Starches and flours used were representative commercially prepared products with the exception of waxy rice starch. This starch was prepared in the laboratory by Dr. S. A. Watson of the Corn Products Refining Company from the same lot of waxy rice flour used in this study.

Starches and flours were mixed with distilled water for the tests described below. For those which produced pastes with pH values below 6.0, measurements were repeated after addition of the amount of sodium hydroxide required to give a cooked paste with a pH value of approximately 6.5.

Hot-paste viscosity and preparation of test samples. To insure uniform gelatinization conditions for the starches in the preparation of all samples for the various tests used, cooking was carried out in a Corn Industries Viscometer, described by Kesler and Bechtel *(14)*. Heating and gelatinization of the starch-water mixture in the stainless steel beaker was accomplished with a glycerol-water bath at 100° C. It was assumed gelatinization at this temperature more closely approximated that obtained under ordinary cooking conditions than would gelatinization with the 92° C. water bath more commonly used with this instrument. Use of a bath of the lower temperature does not provide the heat necessary for maximum swelling of most starches, and hence may give quite different results from those obtained at the higher temperature. The stirrer was run at the standard speed of 24 r.p.m. for the scraper and 60 r.p.m. for the propeller.

The recording mechanism of this instrument provided a complete history of the viscosity of the starch-water mixture. This record formed the basis for determining when gelatinization should be interrupted to provide the samples for further tests. Use of pastes of the same concentration, though preferable, was made impracticable for the present study since, at any concentration chosen, the maximum viscosities of some of the starches lay outside the range of the Corn Industries Viscometer. Therefore, the concentration of each starch was regulated to produce approximately the same maximum viscosity (105 ± 8 g.-cm.). Concentration of the starch in the starch-water mixture was calculated on the dry weight basis. Account was taken of the moisture of the starch, which had been determined by drying in a vacuum oven at 120° C. to constant weight (about 8 hours).

After the proper concentration was found for a given starch, mixtures at this concentration were cooked to each of the following points on the hot-paste viscosity curve: (a) initial rise in the viscosity curve, (b) one-half maximum viscosity, (c) maximum viscosity, (d) maximum viscosity plus 5 minutes, and (e) maximum viscosity plus 20 minutes. When the desired point was reached, the starch-water beaker was quickly removed from the viscometer and the samples poured for the test to be made. Because of the volume of starch paste required for some tests, and also the necessity for avoiding a delay in the preparation of the samples for testing, with resulting "skin" formation in some cases, separate gelatinization was usually carried out for each test at each point of the gelatinization curve.

In the case of the cross-linked starches, which had no maxima in their hot-paste viscosity curves, the point at which the viscosity became nearly constant was arbitrarily selected for comparison with the maximum viscosity value for other starches.

A cooling curve was obtained for each starch by heating the paste 3 minutes after maximum viscosity was reached, then cooling by circulating the aqueous glycerol solution of the heating bath through a copper coil in an ice bath. Cooling was continued for the 40 to 50 minutes required to lower the temperature of the starch paste to 30° C.

Volume of swollen granules. Estimates of the actual volume occupied by the swollen granules at the designated points during gelatinization were made by centrifuging 40-ml. samples of the freshly prepared paste in graduated centrifuge tubes at 3000 r.p.m. for 25 minutes. This length of time was required for a practically constant volume to be obtained. Results are recorded in Table I.

TABLE 1
Gelatinization data

Starch or flour	Concentration of paste (% d.s.)	pH of paste	Hot-paste viscosity (g.-cm.)				Temperature during gelatinization (° C.)					Granular Swelling (% total volume occupied by granules)				
			Maximum viscosity	Maximum viscosity + 5 minutes	Maximum viscosity + 20 minutes	Cooked to maximum viscosity + 5 min., cooled to 30° C.	Initial rise in viscosity	One-half maximum viscosity	Maximum viscosity	Maximum viscosity + 5 minutes	Maximum viscosity + 20 minutes	Initial rise in viscosity	One-half maximum viscosity	Maximum viscosity	Maximum viscosity + 5 minutes	Maximum viscosity + 20 minutes
Unmodified starches																
Potato	1.96	6.6	105	98	49	186	62	70	90	93	95	76	88	100	100	100
Waxy corn	2.98	5.8	105	103	65	139	75	83	89	92	95	96	100	100	100	100
Waxy rice	3.13	6.0	110	110	106	210	67	74	87	91	96	98	100	100	100	100
Waxy sorghum	3.42	5.9	108	105	65	157	77	84	91	93	96	84	100	100	100	100
Tapioca	3.54	6.2	103	95	59	278	65	65	71	82	94	80	87	97	100	100
Arrowroot	4.37	5.6	103	98	85	290	75	75	80	87	96	81	87	97	100	100
Sorghum	4.66	5.5	110	90	73	188	89	89	94	96	97	54	59	84	90	94
Corn	4.90	5.6	113	98	77	148	86	87	91	93	95	56	63	76	82	86
Rice	5.49	6.2	101	95	54	190	89	90	94	96	97	72	90	100	100	100
Sago	5.68	4.3	111	24	0	54	73	73	74	80	94	86	86	92	94	100
Wheat	6.44	6.2	105	80	62	327	89	89	92	94	94	56	62	70	90	96
Flours																
Waxy rice	5.48	6.5	97	52	25		67	68	70	82	92	100	100	100	100	100
Rice	5.57	6.9	100	35	77	397	88	89	93	95	97	52	60	72	85	98
Wheat	9.27	5.7	108	77	5	500	70	74	83	88	95	53	66	83	93	81
Cross-linked starches																
Phosphate cross-linked waxy sorghum	4.11	6.4	106	109	110	226	77	81	95	96	97	67	73	80	81	82
Phosphate cross-linked waxy corn (Stabilizer W-13)	4.15	6.2	103	105	115	207	72	76	93	94	96	66	71	78	81	81
Cross-linked waxy corn (Clearjel)	4.67	6.2	106	110	122	250	73	78	96	97	97	65	70	74	75	74
Thin-boiling starches																
Corn (60-fluidity)	8.26	4.9	111	65	0	222	77	79	85	90	94	64	72	87	100	72
Wheat (59-fluidity) [1]	10.50	5.1	111	85	3	1439	69	74	82	88	94	60	70	85	97	74

[1] Fluidity determined by method used for thin-boiling corn starch.

Cold-paste viscosity. Cold-paste viscosities were determined with a Stormer viscosimeter, the cup and bob of which were modified according to the specifications of E. K. Fischer *(3)* to give true laminar flow. The cup used had a radius of 1.746 cm. and a height of 6.75 cm. The bob had a radius of 1.565 cm. and a height of 3.53 cm. The water bath surrounding the sample cup was maintained at 30° ± 0.5° C. by circulation of water from a large constant temperature bath of that temperature.

An arbitrary test was established for samples cooked to the points on the hot-paste viscosity curves previously indicated. A sample of the freshly cooked paste, slightly larger than the amount needed for the test, was poured into a covered 2-oz. jar which was placed in a 30° C. water bath for 45 minutes (somewhat longer than the time needed to bring the paste to 30° C.). The sample was then given 2 or 3 stirs with a spatula and transferred to the viscosimeter cup. The cup was raised to give a clearance of about 1 cm. between the bottoms of the bob and cup. Sufficient starch paste was used to form an overlay of 5 mm. or more above the bob. Because of the very wide range of viscosities exhibited by different samples in this study, the same falling weight could not be used for all starches. Thus an unavoidable variable in shear stress was introduced. However, the same weight was used for all samples of the same starch. A weight was chosen to give an r.p.m. less than 100, but usually greater than 50, for the sample of the series with the highest cold-paste viscosity. With a few starches which showed extreme variations in viscosity after different cooking times, a weight giving somewhat lower r.p.m. readings with the more viscous samples was used.

A total of 6 readings on each sample was taken in rapid succession, since preliminary experiments had shown that small differences in the length of time between readings did not affect the result. Readings usually increased from the first to the third, but then remained practically constant. For comparison, calculations were made using the fourth reading. Calculation of viscosity in terms of poises was made using the equation

$$\eta = K_v \times \frac{W}{\text{r.p.m.}},$$

where W is the load in grams, r.p.m. is the observed value, and K_v the working constant calculated by Fischer for a cup and bob of the dimensions used.

Gel strength. Tests for gel strength were made with a recording gel tester constructed according to the specifications of E. T. Hjermstad *(6)*. This test, a modification of the much-used method first proposed by Saare and Martens *(10)*, measures the force required to withdraw a disk from a gel in which it is imbedded. The present modification provides for a constant and reproducible increase in the force applied and an automatic recording of the force required to break the gel, using the dynamometer-recorder mechanism of the Corn Industries Viscometer.

Immediately after the sample was cooked to the desired point, it was poured into a 250-ml. Griffin Pyrex beaker. A copper disk, 19 mm. in diameter and 1.5 mm. thick, attached centrally to the bottom of a rod 2.2 mm. in diameter with a hook at the top, was supported level 3.0 cm. below the surface of the gel, and centered in the beaker. A flat washer attached to the rod rested on the center of a slotted cover, holding the disk in position. A thin film of mineral oil was immediately placed on top of the plate. Samples were stored in a refrigerator at about 2° C. for 18 hours. This temperature was chosen in order that the results of this study may be compared with future studies of food products, many of which are too perishable to be held at 25° C., the holding temperature commonly used for tests of starch gels. Since the rate of change in gel strength was found to be insignificant after 18 hours at this temperature, this period was chosen as the holding time. With holding times greater than about 20 hours at this temperature, difficulty was caused by shrinkage of the gel from the beaker walls, allowing the whole gel to be pulled up with the disk during the test.

After the 18-hour holding period, the beaker was clamped in place on the gel-lowering mechanism and the cord from the dynamometer attached to the hook at the end of the disk-rod. With the strongest gels tested, it was necessary to attach an auxiliary weight to the dynamometer arm. The chart drive and gel-lowering mechanism were started and the test made automatically. The yield point of the gel was indicated by a distinct leveling of the recorded curve just before the breaking of the gel.

TABLE 2

Cold-paste viscosity and gel strength

Starch or flour	Concentration of paste (% d.s.)	Cold-paste viscosity (poises)						Gel strength (g.-cm.)				
		Falling weight (g.)	Initial rise in viscosity	One-half maximum viscosity	Maximum viscosity	Maximum viscosity + 5 minutes	Maximum viscosity + 20 minutes	Initial rise in viscosity	One-half maximum viscosity	Maximum viscosity	Maximum viscosity + 5 minutes	Maximum viscosity + 20 minutes
Unmodified starches												
Potato	1.96	150	5.3	5.5	4.6	3.6	1.5	0	0	0	0	0
Waxy corn	2.98	100	1.6	3.1	2.7	2.6	2.7	0	0	0	0	0
Waxy rice	3.13	150	1.2	2.9	2.8	2.6	2.2	0	0	0	0	0
Waxy sorghum	3.42	200	1.1	2.6	3.2	3.3	2.8	0	0	0	0	0
Tapioca	3.54	200	2.4	2.9	4.3	5.4	4.2	0	0	0	0	0
Arrowroot	4.37	200	2.6	3.2	5.8	4.8	5.2	46	61	87	101	115
Sorghum	4.66	200	0.6	0.7	2.3	3.1	6.4	0	12	76	124	136
Corn	4.90	175	0.7	1.3	1.8	2.7	6.4	10	19	52	121	142
Rice	5.49	200	0.8	1.9	4.9	8.6	10.4	0	15	31	71	30
Sago	5.68	200	1.2	1.6	2.1	2.9	3.5	89	100	126	228	312
Wheat	6.44	300	0.7	1.2	4.5	14.9	15.7	41	123	345	520	440
Flours												
Waxy rice	5.48	100	0.5	0.8	2.6	4.7	4.8	0	0	0	0	0
Rice	5.57	225	0.5	0.9	4.9	12.4	14.0	0	0	11	18	15
Wheat	9.27	500	1.1	1.8	4.7	10.8	9.9	5	5	26	116	88
Cross-linked starches												
Phosphate cross-linked waxy sorghum	4.11	175	0.8	1.8	3.3	4.3	5.1	0	0	0	0	0
Phosphate cross-linked waxy corn (Stabilizer W-13)	4.15	175	0.9	2.2	3.6	4.0	4.1	0	0	0	0	0
Cross-linked waxy corn (Clearjel)	4.67	175	1.2	2.1	4.2	4.4	4.8	0	0	0	0	0
Thin-boiling starches												
Corn (60-fluidity)	8.26	350	0.9	1.5	3.8	7.2	7.7	0	32	115	144	210
Wheat (59-fluidity)	10.50	800	1.9	15.9	96.5	220.6	154.4	98	253	434	756	1440

RESULTS AND DISCUSSION

Data presented in Tables 1 and 2 indicate how greatly various properties of the pastes of most natural and modified starches and flours depend upon the extent of cooking which the pastes have undergone. Furthermore, they illustrate the great differences in the concentration of various starches and flours required to produce the same maximum hot-paste viscosity and the variations in the way in which the different starch pastes respond to cooking beyond this point. These differences are large even when the flours and modified starches are ignored and only the natural starches are considered. The unmodified starches are listed in the order of the concentrations used.

It is evident that cooking 20 minutes beyond the attainment of maximum viscosity had little effect upon waxy rice starch, although similar treatment reduced the hot-paste viscosity of the sago starch paste below that which could be measured by the Corn Industries Viscometer (Table 1). The other starches showed effects intermediate between these extremes. The degree of "set-back" which occurred when pastes cooked 3 minutes beyond maximum viscosity were cooled in the viscometer, with stirring, to 30° C. also varied greatly, sago starch again giving the lowest viscosity, but with wheat starch giving the highest. Table 2 shows that when samples for measurement of gel strength and cold-paste viscosity were taken from pastes cooked just to the point of maximum viscosity, these properties were not the same for all samples, as the hot-paste viscosities had been, nor did they vary in relation to the concentration. However, when values for the pastes of unmodified starches cooked 20 minutes beyond maximum viscosity were compared, there appeared to be a nearly direct relationship between the concentration and the cold-paste viscosity, with only one major exception, sago starch. The same appeared true for concentration and gel strength for nearly all the unmodified starches that produced any visible gel structure, the exception in this case being rice starch.

When the logarithm of the cold-paste viscosity for each of the 11 unmodified starches was plotted against its concentration (Figure 1), all points except that for sago starch fell very close to a straight line. Choice of the falling weight for each sample was made to allow use of a single weight for all samples from a single starch rather than for all samples at a particular cooking time. Therefore, values for different starches are not entirely comparable since there were some variations in the weights used. Nevertheless, the conditions do not seem to be sufficiently varied to exclude completely the significance of such a comparison.

That the anomalous behavior of sago starch was not the result of the low pH value of the sample used in these experiments was shown by later studies in which the pH was adjusted to 6.5, without changing the cold-paste viscosity. The fact that the rapid breakdown in viscosity of sago starch paste observed in this study does not seem to have been encountered by Hofstee *(7)* in his studies of gelatinization curves with the V. I. Viscograph suggests that the sample used in this study may possibly have undergone deterioration during the manufacturing process, which is less controlled in the case of sago starch than for other types of starch.

When the logarithm of the gel strength for the 7 gel-forming unmodified starches was plotted against the concentration, only the point for rice starch

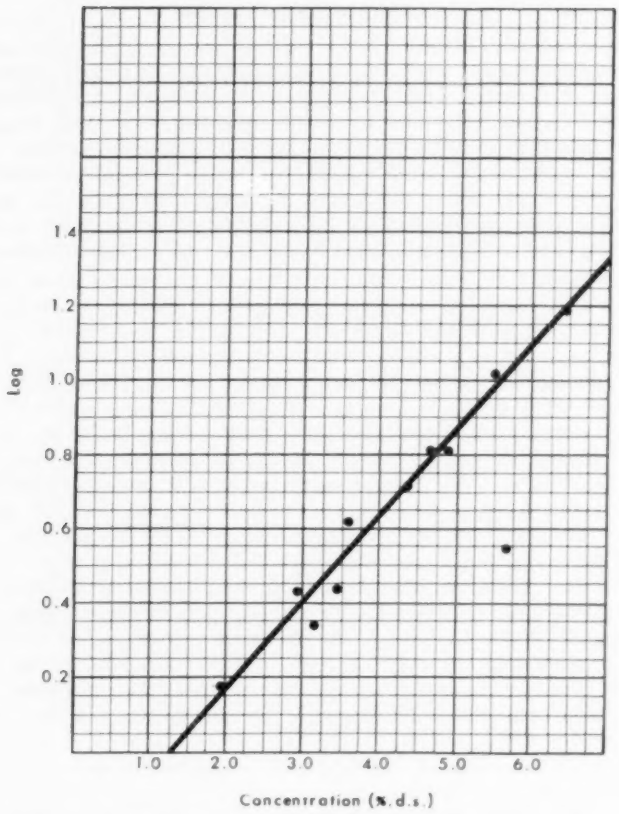

Figure 1. Relation of cold-paste viscosity to concentration.

did not fall close to a straight line (Figure 2) when the pastes which had been cooked 20 minutes beyond maximum viscosity were considered. Possibly the very small size of the rice granules may be a factor in producing this difference.

Many attempts have been made to relate hot-paste viscosity curves to other starch properties. Numerous investigators *(2, 10, 22, 24)* have observed that pastes prepared from the smaller granules of starch from a given botanical species have a higher viscosity than those from the larger granules. Of possibly more significance is the relationship which Hofstee *(7)* pointed out between the hot-paste viscosity curves for several unmodified starches and physical properties reported by other workers. The x-ray diagrams of Katz *(11)*, the water sorption data of Sair and Fetzer *(20)*, the sedimentation volumes of van Itallie *(9)*, and the heats of gelatinization measured by Mullen and Pacsu *(16)* all appear to show that hot-paste viscosity curves reflect to a high degree the intermolecular association within the granules.

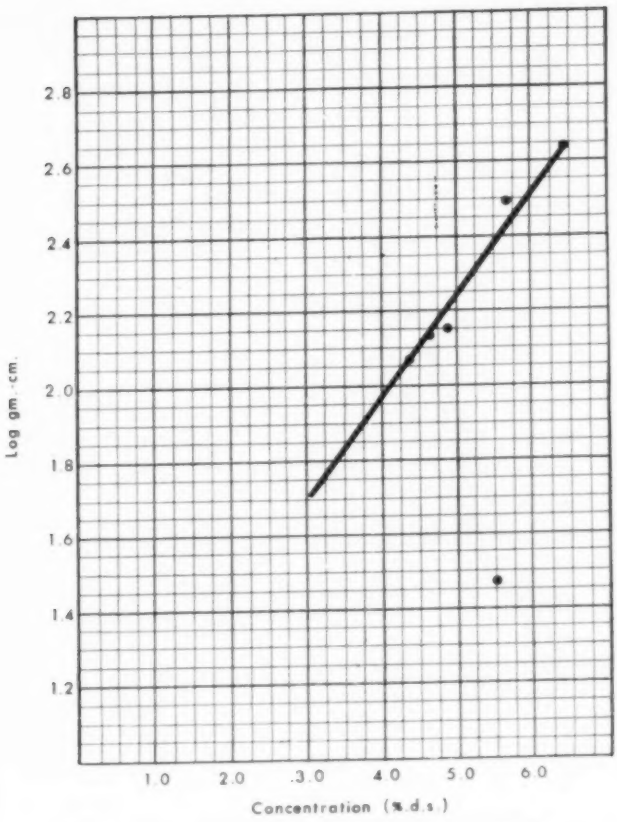

Figure 2. Relation of gel strength to concentration.

Katz's widely accepted interpretation of the hot-paste viscosity curve *(12)* as an overlapping of 2 curves, one caused by the progressive swelling of the starch granules and the other by their breakdown, suggests that in those samples cooked 20 minutes beyond maximum viscosity the intermolecular bonding has been destroyed to a considerable degree. It appears (Figures 1 and 2) that cold-paste viscosity and gel strength of pastes cooked to this extent are determined to a high degree by concentration, regardless of the botanical source of the starch. These results seem to indicate that such factors as ratio of amylose to amylopectin and the size of the amylose molecule *(27, 28)* may be of somewhat minor importance in comparison to bonding within the granule in determining the characteristics of many starch pastes.

In addition to furnishing information for comparing different starches used in foods, these data show how different properties of a single starch are affected simultaneously by altering the time of cooking. For example, as 60-fluidity corn starch in water was cooked beyond the point of maximum

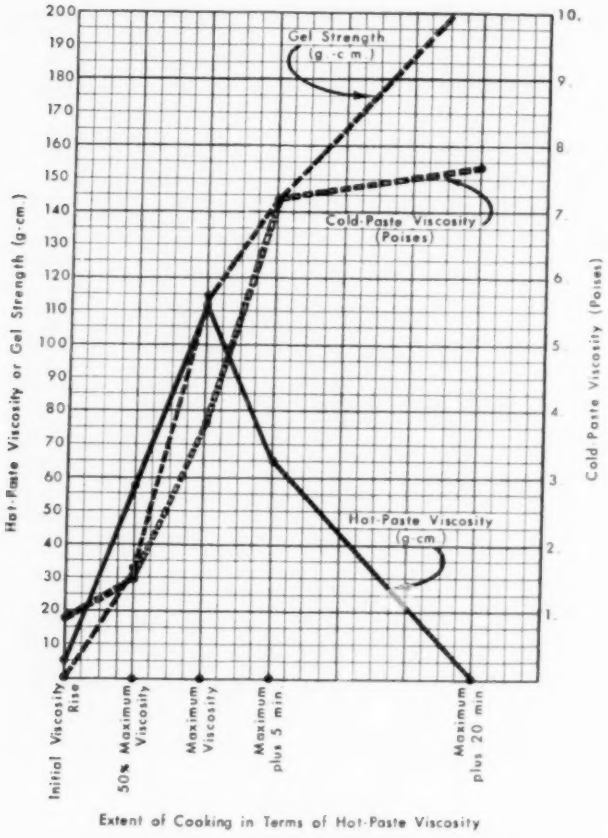

Figure 3. Thin-boiling (60-fluidity) corn starch.

hot-paste viscosity, the mixture thinned very rapidly until the viscosity became too low to register on the viscometer. However, when cooking was stopped at various points and tests made, it was observed that those factors causing gel formation increased very rapidly as the hot-paste viscosity decreased. Cold-paste viscosity also increased, although to a lesser degree. It has been found convenient to plot all the data for a single starch on one graph, as shown for 60-fluidity corn starch in Figure 3, in order to study these simultaneous changes. The resulting graphs illustrate vividly the large differences between starches cooked for shorter periods of time. They also indicate the lack of correlation between hot-paste and cold-paste viscosities, and between gel strength and either of these properties after different cooking times.

Resistance of waxy starch pastes to separation by centrifugation showed that these granules of branched-chain molecules held large amounts of water in very strong association, a fact reflected in the clarity of these pastes. The

lower gelatinization temperatures of these starches as compared with the corresponding non-waxy varieties indicated the greater ease with which water penetrated the waxy granules. These characteristics, as well as the inability of waxy starches to form gels, were presumably caused by the lesser degree of association between the branched molecules.

Introducing cross-linkages into the waxy starches restricts the penetration of water into the granules with the result that the granules swelled only gradually and their pasting curves never passed through a maximum, although the viscosity ultimately reached a nearly constant value. Cold-paste viscosity values likewise increased gradually throughout the cooking period used. Like the parent waxy starches, cross-linked waxy starches did not exhibit the intermolecular forces necessary for gel formation.

The 3 flours studied — wheat, rice, and waxy rice flour — differed considerably in their behaviors from the corresponding starches. There was no consistent difference between the flours and their starches, probably because the other ingredients present, including amylases, varied in kind and amount.

Adjustment of pH value of each starch so that the value after cooking was approximately 6.5 exerted only a very slight effect on the properties measured except in the case of sago starch, which had the lowest pH value when cooked in distilled water without addition of alkali. When the pH of this starch, originally 4.3, was raised to 6.5, the hot-paste viscosity dropped to half its original value, although the gel strength and cold-paste viscosity remained substantially unchanged. Because the effects of these adjustments were so small in all other cases, only the results of the unadjusted systems are reported here.

Behavior of these starches in food products will be modified considerably by other ingredients present. With this present study as a starting point, investigations are being continued on the effects of individual food ingredients and mixtures of ingredients on these systems.

SUMMARY

Hot-paste viscosity, cold-paste viscosity, granular volume, and gel strength are recorded for five points during the cooking of nineteen representative food starches and flours. After prolonged cooking the cold-paste viscosity of the unmodified starches appeared to depend more on concentration than on variety of starch. Likewise, with those unmodified starches which formed gels at the concentrations used, gel strength appeared more dependent upon concentration than upon starch source. However, after shorter cooking periods, no such similarities between the various starches were observed.

Acknowledgments

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A COMPOSITE SAMPLE METHOD FOR THE ANALYSIS OF BLENDED MATERIALS[a]

Many procedures for the bacteriological or chemical analysis of foods involve blending of the sample in order to obtain homogeneous dispersion, suspension and/or thorough extraction of the sample. Since the number of samples examined has frequently been limited by container size, it is often necessary to pool data obtained from several individual analyses or to combine several homogenates for a single determination. A composite sample technic is described here which possesses distinct advantages for certain investigative, quality control and regulatory purposes. One specific application will be detailed and several other uses in the field of bacteriology will be mentioned in order to illustrate the simple principle involved.

An increase in sample size merely necessitates an increase in the size of the container used. Although not perfected[b], a blender with a capacity of one gallon is commercially available[c]. The contents of up to 5 frozen pot pies can be weighed into the gallon container and blended in a 1:5 dilution for composite analysis. (Blending times of 2-5 minutes at low speed or 1-2 minutes at high speed are satisfactory). An "average" count is obtained, therefore, the user must possess background information regarding sample variation in order to most intelligently interpret his results. However, the method should be an aid to those who must examine large numbers of samples for bacteriological quality rather than individual variability. Savings of up to 60% in labor and 80% in materials may be accomplished with little increase of initial investment. For example, military specifications prescribe *(1)* the separate analysis of 2 or 3 samples from every lot considered for purchase. If the composite sample method were used, 5 samples could be combined for one determination, enabling an increase in the number of samples examined with a concomitant decrease in effort expended. The food processor may also find the composite sample method suitable for many of his purposes, particularly when line samples are being taken periodically during a "batch" or plant run. Duplicate samples could be reserved for individual analyses, should results of the composite analysis so indicate.

Use of the large blender technic is not limited to the microbiological analysis of pot pies. The large blender container is suitable for enumeration of the microorganisms on the exterior of whole or cut-up poultry, or on the surface of up to two dozen shell eggs. For such surface counts the foodstuff is placed in a tight-fitting open wire basket *(2)* or on top of a large-mesh wire screen bent around the blender blades in order to protect the food during "washing" (blending).

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[b] Some difficulties were experienced with deformation of the vinyl gasket seal at the top of the container. These difficulties could be minimized by loosening or removing the container top during sterilization.

[c] Model CB-3 Commercial Blendor, Waring Products Corp.

Although the uses cited above are microbiological, the reader may find the composite sample method convenient for other specific applications.

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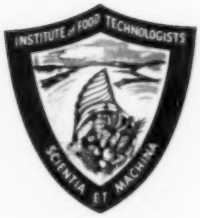
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